J. Biochem. **129**, 61–68 (2001)

Joining of Short DNA Oligonucleotides with Base Pair Mismatches by T4 DNA Ligase[1]

Alexei Cherepanov, Esengül Yildirim, and Simon de Vries[2]

Kluyver Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Received September 11, 2000; accepted October 20, 2000

Oligonucleotide-directed mutagenesis is a widely used method for studying enzymes and improving their properties. The number of mutants that can be obtained with this method is limited by the number of synthetic 25–30mer oligonucleotides containing the mutation mismatch, becoming impracticably large with increasing size of a mutant library. To make this approach more practical, shorter mismatching oligonucleotides (7–12mer) might be employed. However, the introduction of these oligonucleotides in dsDNA poses the problem of sealing a DNA nick containing 5′-terminal base pair mismatches. In the present work we studied the ability of T4 DNA ligase to catalyze this reaction. It was found that T4 DNA ligase effectively joins short oligonucleotides, yielding dsDNA containing up to five adjacent mismatches. The end-joining rate of mismatching oligonucleotides is limited by the formation of the phosphodiester bond, decreasing with an increase in the number of mismatching base pairs at the 5′-end of the oligonucleotide substrate. However, in the case of a 3 bp mismatch, the rate is higher than that obtained with a 2 bp mismatch. Increasing the matching length with the number of mismatching base pairs fixed, or moving the mismatching motif downstream with respect to the joining site increases the rate of ligation. The ligation rate increases with the molar ratio [oligonucleotide:dsDNA]; however, at high excess of the oligonucleotide, inhibition of joining was observed. In conclusion, 9mer oligonucleotides containing a 3 bp mismatch are found optimal substrates to introduce mutations in dsDNA, opening perspectives for the application of T4 DNA ligase in mutagenesis protocols.

Key words: base pair mismatch, DNA ligase, DNA sequencing, mutagenesis, nick-ligation.

The technique of site-directed mutagenesis (SDM) has been applied successfully in many research and industrial applications (*1–3*). However, saturation site-directed mutagenesis, an approach that would produce a library containing all possible single amino-acid replacements in a protein-encoding DNA fragment, is not used in practice, because it is limited by the necessity to synthesize a large number of the 25–30mer oligonucleotides that carry the specific mutations. It is known, that the initial rate of DNA synthesis by DNA polymerases decreases by no more than a factor of two when the length of the complementary priming fragments is reduced from 9–12 to 4–9 nt (*4*). Therefore, it would be possible to employ oligonucleotides as short as 7–12mers to carry out saturation site-directed mutagenesis, which would make this approach applicable in practice. Starting with an ssDNA fragment carrying the gene of interest and a specific primer, a nested set of extensions can be generated by standard methods, producing 3′-hydroxy ends. These 3′-hydroxy ends are subsequently ligated to 7–12mer random oligonucleotides carrying *e.g.* a defined codon at the 5′-end and, finally, DNA synthesis is completed after removal of the non-ligated oligonucleotides (*5*). This approach has a clear advantage over conventional SDM methods. There, in order to introduce a single amino-acid replacement at each position of a protein sequence of, *e.g.*, 500 amino acids, one would require 500 different 30mer oligonucleotides. On the other hand, the same result could be achieved using a 9mer oligonucleotide with a random hexamer binding site and a defined 5′-trinucleotide coding sequence. However, ligation of such an oligonucleotide requires sealing a DNA nick with 5′-terminal base pair mismatches, which is the crucial step in the approach. In the present work, we studied the ability of DNA ligase from the bacteriophage T4 to catalyze this reaction. The number of base pair mismatches and the length of the oligonucleotide substrate were varied as well as the position of the mismatching motif with respect to the joining site.

T4 DNA ligase is the ATP-dependent enzyme that catalyzes the synthesis of the phosphodiester bond between the adjacent 3′-OH and 5′-PO_4 functions of two DNA fragments annealed to the DNA template (*6*). Under certain conditions T4 DNA ligase is able to join two double-stranded DNA fragments (blunt-end ligation) (*7–10*); the enzyme can

[1] This work was supported by the Association of Biotechnology Centers in The Netherlands (ABON) (Project I.2.8) and in part by the Netherlands Research Council for Chemical Sciences (CW), with financial aid from the Netherlands Technology Foundation (STW) (grant 349-3565).

[2] To whom correspondence should be addressed. Phone: +31-15-2785139, Fax: +31-15-2782355, E-mail: S.deVries@tnw.tudelft.nl

Abbreviation: TP, template-primer dsDNA.

Enzymes: DNA ligase [EC 6.5.1.1], RNA ligase [EC 6.5.1.3].

also reverse the ligation reaction by creating nicks in dsDNA (*11*). T4 DNA ligase is known for its ability to join DNA fragments containing Watson-Crick base pair mismatches (*12–16*) and oligonucleotides as short as tetramers (*17–19*). Results from (*13*) indicate that T4 DNA ligase can join longer DNA fragments containing several mismatching base pairs 3–5 nt away from the joining site, either upstream or downstream. Summarizing the literature data on the substrate specificity of T4 DNA ligase, one can conclude that the enzyme is able to join short DNA oligonucleotides and to tolerate Watson-Crick base pair mismatches for the longer DNA fragments.

The goal of the present work was to study whether T4 DNA ligase can combine these two abilities, *i.e.* to catalyze the effective joining of short (7–12mer) DNA oligonucleotides, containing several base pair mismatches. This work was performed as part of a program to develop new mutagenesis methodologies.

MATERIALS AND METHODS

Enzymes and Oligonucleotides—MBI Fermentas (Lithuania) supplied T4 DNA and RNA ligases. Cy-5 (Dye 667, #27-1801-02)-5′–labeled fluorescent primer A (Fig. 1) was obtained from Amersham-Pharmacia Biotech. Other DNA oligonucleotides were HPLC pure and supplied by Eurogentec. Additional PAGE purification of synthetic oligonucleotides was performed according to (*20*).

Ligation Conditions—The ligation was performed 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 incubation temperature and concentrations of oligonucleotide substrates were varied as indicated in the text. To follow the formation of the adenylylated DNA intermediate, α-^{32}P–labeled ATP was used. The concentration of TP was 1 μM, the amount of enzyme added between 0.05–1 unit (yielding a final concentration of 20 nM–0.4 μM). Nicked dsDNA was prepared by mixing the 72mer template B and Cy-5–labeled 24mer oligonucleotide A in a 1:1 ratio with the desired excess of 3′-oligonucleotide in 5 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$ followed by incubation for 10 min at 65°C, 10 min at 37°C, 3–5 min at RT, and finally for 10–15 min at 4°C. Difference UV-VIS spectroscopy showed that binding of the 24mer oligonucleotide to the 72mer DNA template was stoichiometric and that virtually no free template was present under 1:1 conditions, since addition of extra oligonucleotide no longer induced a hypochromic effect at 260 nm. The ligation buffer was added after preparation of dsDNA and before addition of the enzyme to avoid unnecessary decomposition of DTE and ATP. The reaction was initiated by addition of the enzyme. At intervals, 0.3 μl aliquots were withdrawn from the reaction mixture and mixed into 10 μl of 100% formamide, 10 mM NaOH, 10 mM EDTA, pH 9.5, 5 mg/ml of Blue Dextran. Samples were taken after 15 s, 50 s, 2 min, 5, 15, 45, 90, 180, 300, 1,440, and 2,440 min.

Synthesis and Purification of the Adenylylated DNA Oligonucleotides—5′-Phosphorylated DNA oligonucleotide (50 nmol) was incubated for three hours at room temperature in a volume of 0.5 ml in the presence of 70 units of T4 RNA ligase. The reaction buffer contained 2 mM ATP, 0.05 mg/ml BSA, 10 mM DTT, 10 mM $MgCl_2$, and 50 mM HEPES-NaOH (pH 8.0 at 20°C). The reaction was stopped by phenol–chloroform extraction. Triethylammonium acetate (TEAA, pH 7) was added to the supernatant to a final concentration of 0.2 M and the solution was loaded onto a 50 ml Nova-Pak C18 reverse phase cartridge pre-equilibrated with 0.1 M TEAA and 6% acetonitrile. Elution was performed with a 6–15% acetonitrile gradient. Fractions containing the adenylylated oligonucleotide were collected and lyophilized to dryness several times from a water-methanol solution.

Sequencing of Ligation Products—Maxam-Gilbert sequencing of ligation products was performed according to (*20*), except that the incubation times with DNA-modifying agents (except piperidine) were 10-fold longer and Cy-5 labeled DNA was used instead of ^{32}P-labeled DNA. For A and A + G modification reactions, 1 μl (~0.5–1 pmol) of ligation product was taken from the reaction mixture without preliminary purification from the protein material and buffer exchange. For the cleavage at C and C + T sites, 1.5–3 pmol of fluorescent DNA was used.

Comments on Maxam-Gilbert Sequencing of 5′-Cy 5 Labeled DNA—The Cy-5 group showed reasonable stability towards most chemical reagents used in the Maxam-Gilbert protocol. The cleavage of Cy-5-labeled DNA at G-sites (dimethyl sulfate) or at A + G sites (piperidine formate) was especially effective; almost no fluorescence was lost

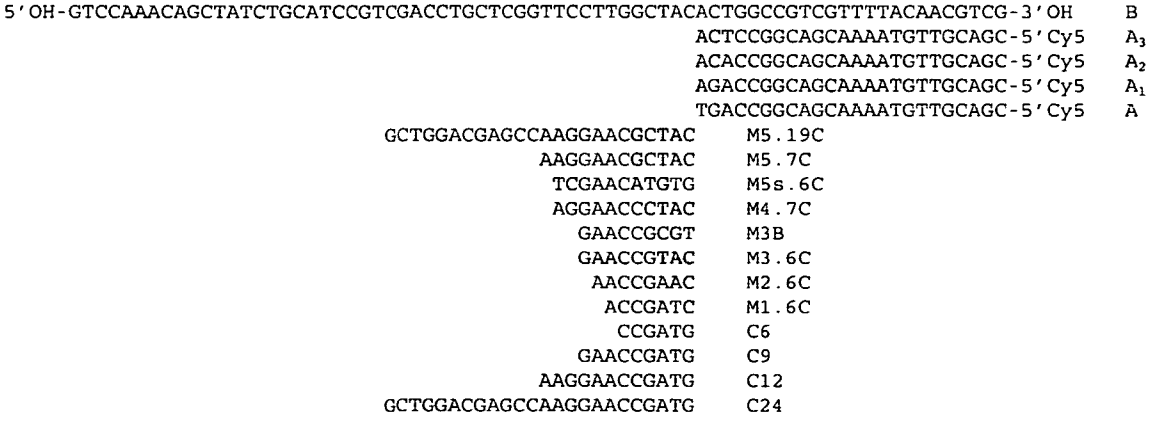

Fig. 1. **The model system to study DNA joining catalyzed by T4 DNA ligase.** Oligonucleotides A-A_3 have a Cy-5 fluorescent label at the 5′-end. DNA oligonucleotides except A-A_3 and B are 5′-phosphorylated. Mismatching base pairs are shown in gray.

after cleavage. Modification of DNA with hydrazine (cleavage at C or C + T) resulted in the loss of approximately half of the fluorescence during the course of incubation; to compensate for this, gels were loaded with 2 times more DNA than used for the A or A + G reactions. The modification of DNA with NaOH (A > C) did not work in our hands; it seems that prolonged incubation under alkaline conditions degrades the Cy-5 label; incubation of labeled DNA with NaOH for shorter periods of time is apparently not sufficient to modify DNA.

Separation of Ligation Products—Separation of the DNA fragments, including sequenced DNA, was performed on an ALF Express DNA sequencer (Amersham-Pharmacia) using 6 to 17% acrylamide gels with 7 M urea in TBE buffer for gel electrophoresis. Runs were performed at 55°C, with a current of 80 mA.

Processing of Experimental Data—The chromatographic separation of Cy-5–labeled DNA for each data point yielded the fluorescence profiles of the starting material and ligation product. Separation of ^{32}P-labeled DNA was visualized using a Molecular Dynamics Phosphorimager SI (Molecular Dynamics) and quantified using ImageQuant software. Data obtained from both Cy5- and ^{32}P-labeled material was imported into Igor Pro v. 3.14 (WaveMetrics); further data processing was performed in this software package. In each case the amount of ligation product, P, was determined as $P = V_P{}^*S_0/(V_P + V_S)$, where V_P, V_S are the areas of the product and starting material peaks, respectively, and S_0 is the initial amount of substrate in the assay. The values for the initial ligation rate were determined as $V_{init} = f'(t)$ at $t = 0$, where $f(t)$ is a double exponential fitting function.

RESULTS AND DISCUSSION

The Model System—It was shown recently that protein–DNA contacts in the DNA binding site of DNA ligase from the bacteriophage T7 extend only as far as 3–5 nt upstream and 7–9 nt downstream of the nick (*21*). T7 DNA ligase is a representative member of the ATP-dependent family of DNA ligases and therefore we could expect similar nick recognition by DNA ligase from bacteriophage T4. The relatively short extension of the protein–DNA contacts at either side of the nick suggests that the ligase specificity does not change significantly with increasing length of the TP dsDNA; *i.e.* that the effectiveness of ligation of a particular oligonucleotide annealed either to short or long dsDNA will be similar. Therefore, we used a 72:24mer dsDNA substrate (Fig. 1) as a model system to study the kinetics of ligation of short mismatching oligonucleotides to long dsDNA.

To allow easy detection and quantification of the products of the joining reaction, 24mer 5′-oligonucleotides were labeled with a fluorescent Cy-5 label (5′- refers to the fact that the oligonucleotide is located upstream with respect to the position of the nick). Some of these oligonucleotides (A_1–A_3) contained 1 to 3 adjacent base pair mismatches at the 3′-end, *i.e.* upstream with respect to the joining site. The 3′-oligonucleotide substrates (annealed to the template downstream of the joining site) differed in length (6–24mer) and contained several base pair mismatches. Eight possible Watson-Crick base pair mismatches were present in different combinations, including so-called stable mismatches G·T, G·A and G·G (*22–25*), intermediately stable mismatches A·C, A·A, and T·T (*25, 26*), as well as unstable C·C and C·T mismatches (*25, 27*). Ligations with complementary 3′-oligonucleotides served as controls (Fig. 1; C6, C9, C12, and C24).

Determination of the Sequence of the Ligation Products—The length and sequence of the joining products of the mismatching 3′-oligonucleotide substrates were determined to assure that no insertion or deletion of DNA fragments takes place during ligation. The length was determined using appropriate DNA markers and in all cases corresponded to the calculated sum of the lengths of the joined 3′- and 5′-oligonucleotides.

To verify the presence of the expected mismatching motifs, the sequences of the ligation products were determined using the Maxam-Gilbert sequencing method. As an example, the sequencing ladder of the 35mer ligation product is presented, obtained after joining the 11mer M5s.6C

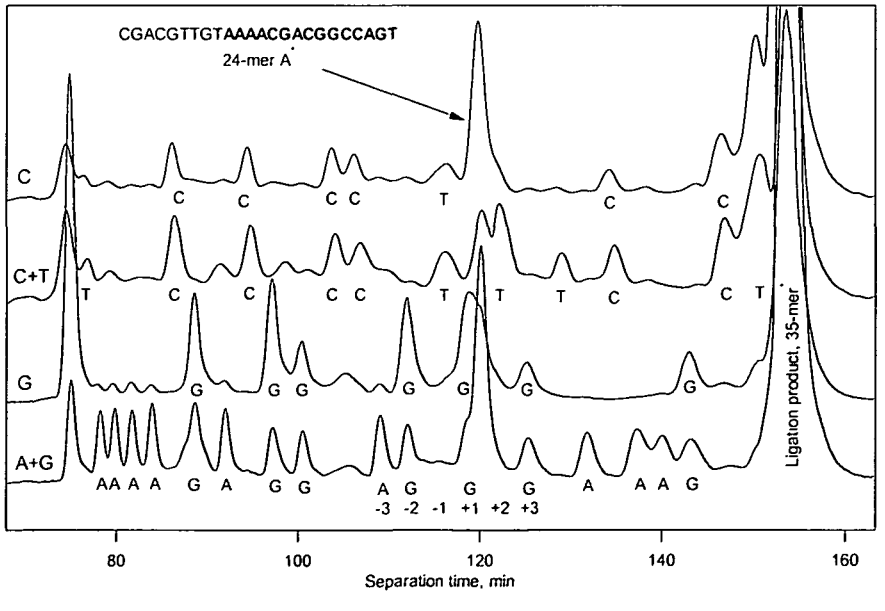

Fig. 2. **Maxam-Gilbert sequencing of a ligation product containing base pair mismatches.** 11mer M5s.6C was joined to the to the 24mer A as described in "MATERIALS AND METHODS." The numbers above the sequence indicate the position of the nucleotide with respect to the joining site. °The starting material and ligation product that are not affected by the modifying reagents indicate partial conversion. Due to the removal of bases by piperidine treatment, modified DNA fragments migrate in the gel faster than non-modified fragments of the same length.

to the 24mer A (Fig. 2). In total, oligonucleotide M5s.6C contains 5 base pair mismatches, with the GTA/TCG mismatching fragment located 2 nt downstream of the joining site and the CT/CT fragment—at the 3′-end of the 5′-oligonucleotide. Both fragments were found in the 35mer-ligation product, verifying the ability of the T4 DNA ligase to join short DNA oligonucleotides with several base pair mismatches in proximity of the joining site.

Characteristic Features of Joining Short DNA Oligonucleotides—Joining a short oligonucleotide to a long dsDNA with single-stranded 5′-overhangs differs from sealing a nick in a long dsDNA because the oligonucleotide is able to dissociate from the catalytic complex [ligase:TP:oligonucleotide] during enzyme turnover. Only part of the TP is saturated when the amount of oligonucleotide in the reaction mixture is stoichiometric with respect to the amount of TP DNA. As a result, the ligation rate is lower than its maximal value. An increase in the 3′-oligonucleotide:TP ratio increases the rate; however, a large excess of the former inhibits joining, and the longer the oligonucleotide, the stronger the inhibition (Table I, C06-C24). In Fig. 3 these issues are addressed employing short mismatching as well as complementary oligonucleotide substrates. The absolute joining rates shown in this figure differ by more than four orders of magnitude (Table I). Therefore, each curve in the graph was normalized taking a joining rate equal to unity at a 1:1 added ratio of 3′-oligonucleotide:TP. At a 2:1 ratio, a small decrease in the ligation rate was observed in the case of the complementary 24mer C24, indicating that the catalytically competent dsDNA substrate is formed stoichiometrically at the assay temperature (+4°C), a finding confirmed by difference UV-VIS spectroscopy (not shown). The complementary hexamer C6, however, is joined with a maximal rate at a 3:1 ratio, indicating its ability to dissociate from the TP. Further increases in the C6:TP ratio lead to a decrease in the joining rate. The mismatching 3′-substrates are joined optimally at higher ratios, *i.e.* at a 30–100:1 over TP, indicating, among other things, that the dissociation constants of these oligonucleotides for the TP or [TP:enzyme] complex are higher than that of the complementary hexamer C6.

The fact that short oligonucleotides dissociate from the catalytic ligase-DNA complex is also illustrated by the accumulation of the adenylylated intermediate during the joining reaction. For example, when a 30-fold excess of the mismatching 12mer M5.7C (containing 7 complementary base pairs) is joined, virtually all of the free 12mer is adenylylated before one-fifth of the ligation product is formed due to dissociation from the [TP:oligonucleotide:enzyme] complex. However, no excess of the adenylylated intermediate is formed when a 30-fold excess of the mismatching 24mer M5.19C (19 complementary nucleotides) is joined to the TP under the same conditions (Fig. 4), since it apparently does not dissociate from the TP at 4°C.

The Rate-Limiting Step of Joining—The data in Table I indicate that the rate of joining of complementary oligonucleotide substrates at a 1:1 oligonucleotide:TP ratio decreases nearly 1.5-fold upon increasing their lengths from 6- to 24mer, in agreement with the result that T4 DNA ligase displays maximal joining rates at the melting temperature of the duplex DNA substrate (*28*). The shorter the 3′-substrate, the closer its melting temperature is to the incubation temperature (4°C), and, consequently, the

TABLE I. **Parameters of the T4 DNA ligase-catalyzed joining of complementary and mismatching oligonucleotides.** All reactions were carried out at 4°C in the presence of 1 mM ATP using a 30:1 oligonucleotide:TP ratio. Adenylylated oligonucleotide substrate M1.6C-AMP was prepared as described in "MATERIALS AND METHODS." A 10-fold molar excess of T4 RNA ligase with respect to DNA ligase was used.

Substrate	Initial turnover (h^{-1})		Extent of ligation (relative %)	
	+ RNAlig.	−RNAlig.	+ RNAlig.	−RNAlig.
A:C6	710	990 (860[d])	85–90	
A:C9	380	520 (720[d])		
A:C12	350	430 (710[d])		
A:C24	180	260 (610[d])		
A:M1.6C	35	19	75–85	
A:M1.6C-AMP[a]	7.1	4.5		
A:M1.6C-AMP[b]	32	18		
A:M2.6C	12	5		
A:M3B	20	13		
A:M3.6C	31	24	85–90	
A:M4.7C	1.3	0.71	80	75
A:M5.7C[c]	Nd	96	Nd	92
A:M5.7C	0.16	0.13	65	20
A:M5.19C[c]	Nd	60	Nd	95
A:M5.19C	3.8	2.3	70	45
A:M5s.6C	78	45	85–90	
A_1:C6	0.022	0.007	70[e]	40[e]
A_2:C6		~0		

Pre-adenylylated oligonucleotide M1.6C-AMP was joined both in the [a]presence and [b]absence of ATP. [c]Rate and yield of formation of the adenylylated 3′-oligonucleotide. [d]Ligation at 1:1 oligonucleotide:TP ratio. [e]yields obtained after 4 days of incubation.

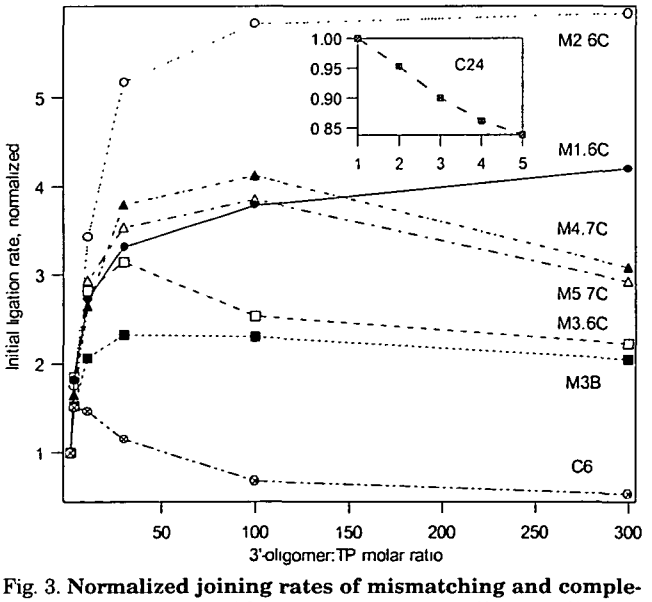

Fig. 3. **Normalized joining rates of mismatching and complementary oligonucleotides determined at different oligonucleotide:TP ratios.** Ligation was performed with 1 μM of the 72/24mer B:A at 4°C as described in "MATERIALS AND METHODS." The excess of 3′-oligonucleotide is indicated on the abscissa of the graph. Joining rates of oligonucleotide C24 are shown in the insert. Concentration of T4 DNA ligase was 0.4 μM in the case of mismatching 3′-oligonucleotides and 6 nM in the case of complementary oligonucleotides C6 and C24. Initial turnover values in h^{-1} at 1:1 oligonucleotide:TP ratio were: C06–860; C24–610; M1.6C–5.7; M2.6C–0.96; M3B–5.6; M3.6C–7.6; M4.7C–0.18; M5.7C–0.037. The error in the rate determination did not exceed 10% of the absolute value.

higher the ligation rate. However, this correlation is opposite when mismatching 3′-oligonucleotides are joined (Table I, compare values for M5.7C and M5.19C), indicating that the rate-limiting step of joining may be different for complementary and mismatching 3′-oligonucleotides.

From the data in Table I we conclude that the joining rate of oligonucleotides with mismatches downstream of the nick is limited by the actual formation of the phosphodiester bond. For example, the adenylylation rate of the 24mer M5.19C is more than 10-fold faster than its joining rate, whereas the 12mer M5.7C is adenylylated more than 500-fold faster. In addition, the ATP-independent rate of joining of the adenylylated intermediate AMP-M1.6C is essentially as fast as the ATP-dependent rate of joining of the non-adenylylated oligonucleotide M1.6C. The fact that the rate of joining of AMP-M1.6C in the presence of ATP is about one-fourth that in its absence is due to T4 DNA ligase being mainly in the adenylylated form, and so not capable of binding/joining an adenylylated oligonucleotide *cf.* (*29*).

On the other hand, the steady-state concentration of the adenylylated intermediate during ligation of complementary 3′-oligonucleotide substrates is very low, in fact, similar to the concentration of the enzyme (not shown) *cf.* (*29*). This implies that either the adenylylation of the 3′-oligonucleotide is rate limiting, or that the enzyme does not dissociate from the dsDNA after adenylylation of the oligonucleotide before sealing the nick. The rate of adenylylation of the 12mer M5.7C is higher than of the 24mer M5.19C, and so is the joining rate of the complementary 12mer C12 in comparison to the 24mer C24 (Table I). This could indicate that, in contrast to the case of mismatching oligonucleotides, adenylylation of the DNA is the major rate-limiting step in the sealing of the complementary nick. On the other hand, the existence of a stable "S-complex" [enzyme:TP:oligonucleotide-AMP] (*30*) indicates a high affinity of the non-adenylylated enzyme for the adenylylated DNA nick, which could also explain the low concentration of the DNA–AMP intermediate. This would suggest that the actual rate of joining is limiting, just as in the case of mismatching oligonucleotides.

Joining of Mismatching Oligonucleotides—Joining of mismatching oligonucleotides by T4 DNA ligase is sensitive to the position of the mismatching motif with respect to the joining site (*16*). We observed that T4 DNA ligase joins complementary 5′-oligonucleotides to 3′-oligonucleotide sub-

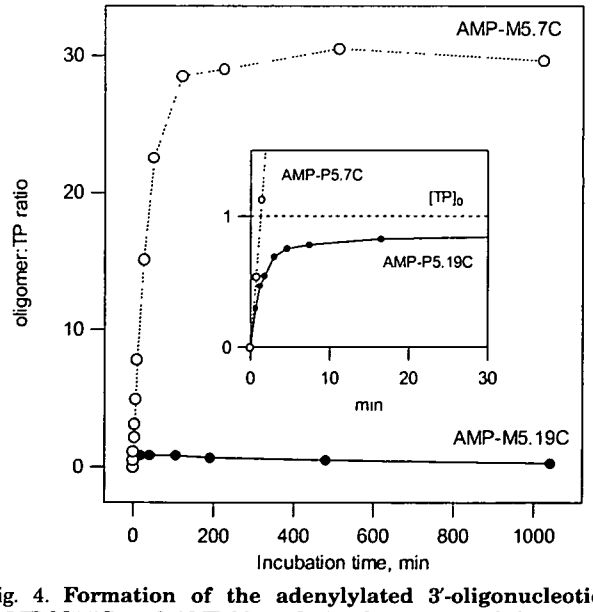

Fig. 4. **Formation of the adenylylated 3′-oligonucleotides (AMP-M5.7C and AMP-M5.19C) in the course of the joining reaction.** Adenylylation of M5.7C (30 μM, open circles, dotted lines) or M5.19C (30 μM, filled circles, solid lines) annealed to the 72/24mer TP B:A (1 μM) by T4 DNA ligase (0.4 μM) in the presence of 1 mM ATP at 4°C. A blow-up of the first 30-min-interval is shown in the insert. The total amount of TP in the reaction mixture (1 μM) corresponds to 2.5 turnovers of the enzyme (0.4 μM) and is indicated in the insert by the dashed line.

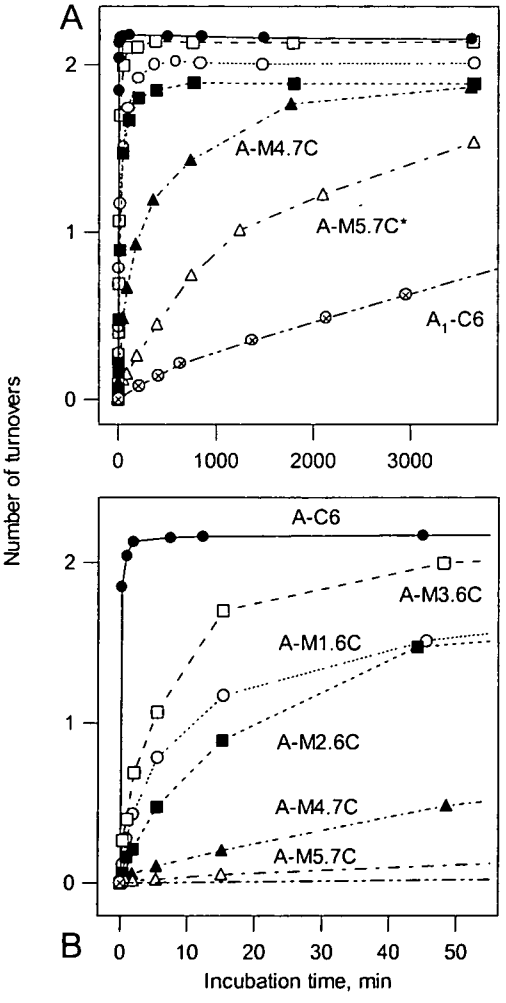

Fig. 5. **Ligation of 3′-oligonucleotides by T4 DNA ligase: product formation curves.** Panel A: The 24mer A was ligated to the 3′-oligonucleotide C6 (filled circles), M1.6C (open circles), M2.6C (filled squares), M3.6C (open squares), M4.7C (filled triangles), M5.7C (open triangles); 5′-oligonucleotide A_1 was ligated to the 3′-oligonucleotide C6 (crossed open circles). Ligation was performed at 4°C, with 1 unit (~4 pmol) of enzyme (0.4 μM final concentration) as described in "MATERIALS AND METHODS." (*) In the case of M5.7C, the ligation product relates to the sum of all ligation products formed in the course of joining (36mer and 48mer). Ligation was performed in the presence of a 10-fold molar excess of T4 RNA ligase over T4 DNA ligase in the case of the joining of oligomer A_1 to C6. A blow-up of the first 50-min-interval is shown in the panel B.

strates with mismatches (downstream with respect to the nick) far more readily than complementary 3′-oligonucleotides to mismatching 5′-oligonucleotides (mismatches upstream with respect to the nick) (24mer A_1, Table I, Fig. 5). dsDNA substrates containing two or three adjacent mismatches upstream of the nick apparently do not ligate at all.

The rate and extent of ligation decreases with increasing length of the mismatching motif at the 5′-end of the 5′-oligonucleotide (Fig. 5, Table I). Unexpectedly, 3′-oligonucleotides containing three adjacent mismatching base pairs (CAT/CAT, 9mer M3.6C or, to the lesser extent CAT/TGC, 9mer M3B) break this trend. In this case the value of the initial joining rate is 4.8- or 2.5-fold, respectively, higher than obtained for ligation of the 3′-oligonucleotide with 2 terminal mismatches, M2.6C. The 2-fold difference in joining rates between M3.6C and M3B indicates that the ligation rate is dependent on both the number of mismatches and the nucleotide composition of the mismatching motif. For example, the 9mer M3.6C contains one unstable C·C mismatch, while M3B has two unstable C·T mismatches.

The addition of T4 RNA ligase slightly inhibits the joining of complementary 3′-oligonucleotides, but stimulates the rate of ligation of the 3′-oligonucleotides with mismatches, increasing both the rate and yield of joining. This finding is similar to that previously reported for blunt-end ligation (*9*), even though the stimulatory effect in our case is less pronounced. The mechanism of stimulation is unclear; however, the explanation that T4 RNA ligase may accelerate the reaction by (rapid) adenylylation of the DNA substrate or that it protects T4 DNA ligase non-specifically against inactivation upon dilution can be eliminated. Even in the absence of ATP, T4 RNA ligase stimulates the rate of joining of pre-adenylylated 3′-oligonucleotide substrates with mismatches (Table I). Note that BSA is present as a protecting agent in both assays.

Analysis of the values in Table I shows that the 3′-oligonucleotides containing a 3 bp mismatch would be the most convenient to use in mutagenesis protocols. First, the joining rate and extent of ligation are similar to the oligonucleotides with 1–2 bp mismatches. Second, a 1–2 bp mismatch is not sufficient to introduce a desired single amino acid replacement in a random fashion. On the other hand, the joining of oligonucleotides containing 4–5 bp mismatches is so slow that even in the presence of T4 RNA ligase their applicability seems *a priori* limited. In addition, the efficiency of ligation of oligonucleotide substrates containing a 3 bp mismatch can be improved significantly when we place the mismatching fragment 2 nt away from the joining site (as in case of M5.6s). However, in this case one would have to use a 16-fold (4^2) higher concentration of such oligonucleotide substrate for random mutagenesis, which may lower the yield by inhibiting the joining.

Head-to-Tail Ligation of Mismatching Oligonucleotides—The low fidelity of *in vitro* nick-sealing activity of T4 DNA ligase is further demonstrated by head-to-tail joining of mismatching 3′-oligonucleotide substrates. Ligation products of different length are formed when the 12mer M5.7C with 5 adjacent base pair mismatches is joined to the 72/24meric dsDNA, yielding dsDNA with several non-complementary fragments. Comparison of the joining products of the PAGE-purified M5.7C with the non-purified commercial preparation shows that the -1 and -2 oligonucleotides, which are present in the commercial sample, are also joined by this enzyme (Fig. 6). Together with the single ligation product (total length 36), we found that DNA fragments of higher molecular mass corresponding to double (48) and trace amounts of triple (60) ligation products were synthesized. According to the sequence, the second M5.7C oligonucleotide in the double ligation product in total contains seven mismatching and only five matching base pairs, distributed in 1-, 3-, and 1-nt-long fragments (Fig. 6). Among the mismatching base pairs are three stable G·A mismatches, two intermediately stable A·C mismatches

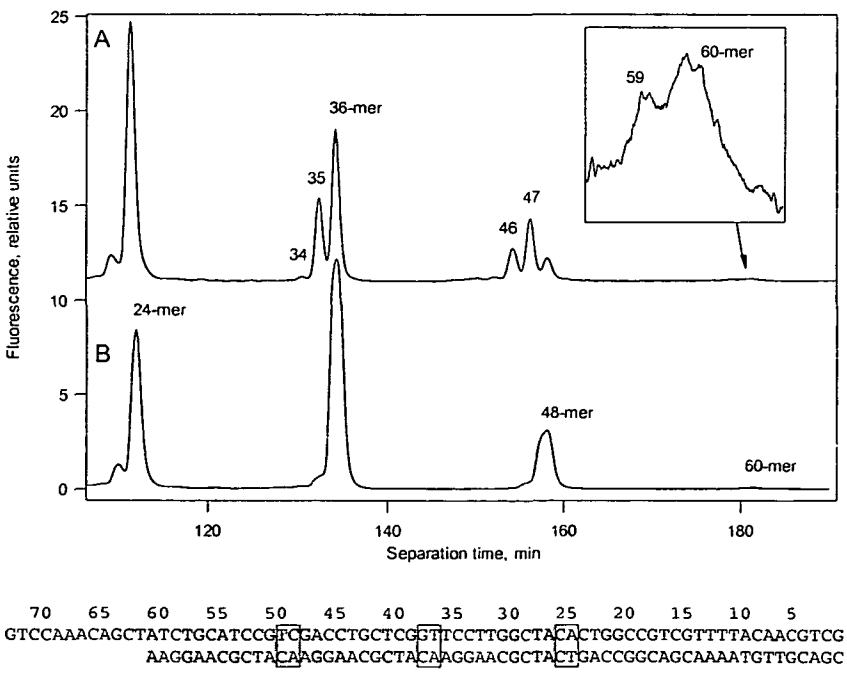

Fig. 6. **Ligation of HPLC-pure 12mer M5.7 before (A) and after PAGE purification (B).** Aligned sequences of the 72mer template B and 60mer ligation product are shown below. Mismatching base pairs are shown in gray; joining sites are shown in boxes.

and two unstable C·T mismatches. The rate of formation of the 48mer strongly depends on the molar ratio M5.7C:TP (Fig. 7). At a 1:1 3′-oligonucleotide:dsDNA ratio only a negligibly small amount of 48mer is formed within the incubation time of ~7 hours (~0.6% of the total amount of substrate). Whereas at a 100:1 ratio of M5.7C:TP as much as 3 pmol or about 20% of the 48mer is synthesized. A ratio of 100:1 3′-oligomer:dsDNA ratio is apparently optimal for the head-to-tail condensation of M5.7C, whereas a 30:1 ratio is optimal for the formation of the 36mer product. At a 300:1 ratio we observed inhibition of ligation, leading to a decrease in the total ligation yield and the disappearance of the 60mer joining product.

The results shown in Fig. 7 indicate that the number of closely located mismatching fragments that can be introduced in the same dsDNA molecule by T4 DNA ligase using a non-degenerate oligonucleotide is limited. The yield of the double ligation product is low (~20–30%), whereas the amount of triple ligation product formed is negligible. In addition, only the 12mer M5.7C and not other oligonucleotide substrates (M1.6C, M2.6C, *etc.*) joins head-to-tail, indicating that both the length of the 3′-substrate and the sequence of the DNA template at the site of joining play an important role in the oligomerization.

Summarizing, we have demonstrated that T4 DNA ligase is capable of joining 7–12mer DNA oligonucleotides containing in some cases up to 7 base pair mismatches, and that the rate of formation of the phosphodiester bond appears to be the major determinant of the overall rate of catalysis. 9Mer oligonucleotides containing a 3 nt-long mismatching motif are found to be an optimal compromise between the number of adjacent mismatching base pairs that can be incorporated into dsDNA and the effectiveness of the ligation reaction. In principle, T4 DNA ligase is capable of sealing a nick between two closely located mismatching motifs, as indicated by the head-to-tail joining reaction. The observed *in vitro* mismatch-joining activity of T4 DNA ligase could be applied to mutagenesis protocols to introduce mutations in dsDNA.

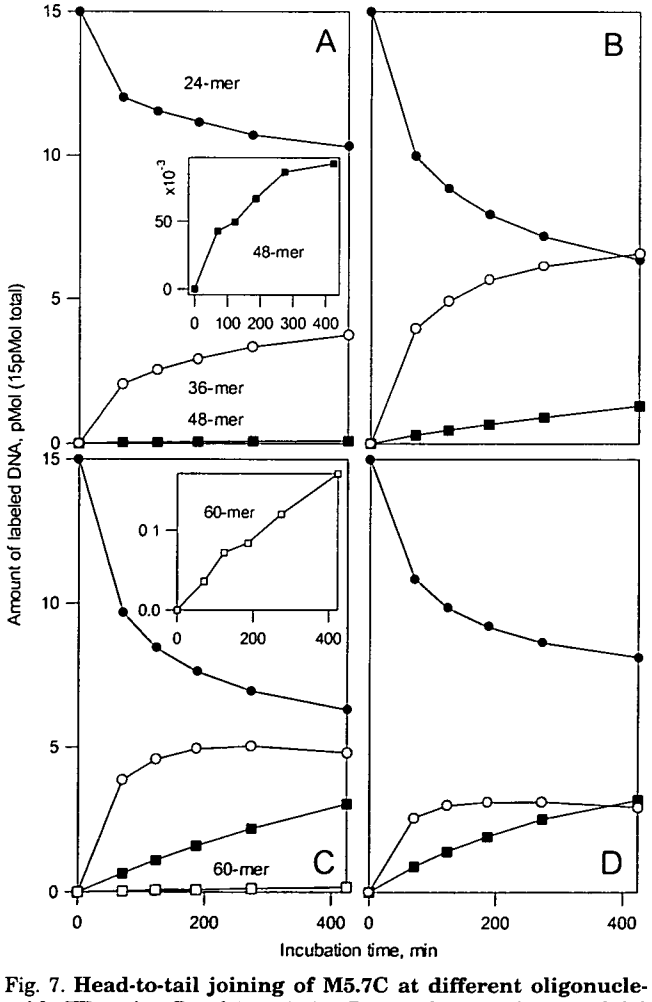

Fig. 7. **Head-to-tail joining of M5.7C at different oligonucleotide:TP ratios.** Panel A, ratio 3:1; B, 30:1; C, 100:1; D, 300:1. Solid circles, non-joined oligonucleotide C; open circles, 36mer ligation product C-M5.7C; solid squares, 48mer product C-(M5.7C)$_2$; open squares, 60mer product C-(M5.7C)$_3$. Joining was performed at 4°C using 3.1 units of T4 DNA ligase (~12 pmol).

The authors thank Dr. Z. Debyser and Dr. P. Cherepanov for their kind assistance in using the ^{32}P handling and detection facilities of the Rega Institute of Medical Research, Leuven, Belgium. We thank Prof. W.R. Hagen for critically reading the manuscript.

REFERENCES

1. Ishii, T.M., Zerr, P., Xia, X.M., Bond, C.T., Maylie, J., and Adelman, J.P. (1998) Site-directed mutagenesis. *Methods Enzymol.* **293**, 53–71
2. Ling, M.M. and Robinson, B.H. (1997) Approaches to DNA mutagenesis: an overview. *Anal. Biochem.* **254**, 157–178
3. Botstein, D. and Shortle, D. (1985) Strategies and applications of in vitro mutagenesis. *Science* **229**, 1193–1201
4. Nevinsky, G.A., Veniaminova, A.G., Levina, A.S., Podust, V.N., Lavrik, O.I., and Holler, E. (1990) Structure-function analysis of mononucleotides and short oligonucleotides in the priming of enzymatic DNA synthesis. *Biochemistry* **29**, 1200–1207
5. Schulte, A.E., Yildirim, E., Cherepanov, A., Kramer, E.H.M., and de Vries, S. (1999) Development and application of a novel method for saturated random mutagenesis in *4th International Symposium on Biocatalysis and Biotransformations*, pp. P2444, Giardini Naxos-Taormina, Italy
6. Weiss, B., Jacquemin-Sablon, A., Live, T.R., Fareed, G.C., and Richardson, C.C. (1968) Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. *J. Biol. Chem.* **243**, 4543–4555
7. Sgaramella, V. (1972) Enzymatic oligomerization of bacteriophage P22 DNA and of linear Simian virus 40 DNA. *Proc. Natl. Acad. Sci. USA* **69**, 3389–3393
8. Sgaramella, V., Van de Sande, J.H., and Khorana, H.G. (1970) Studies on polynucleotides, C. A novel joining reaction catalyzed by the T4-polynucleotide ligase. *Proc. Natl. Acad. Sci. USA* **67**, 1468–1475
9. Sugino, A., Goodman, H.M., Heyneker, H.L., Shine, J., Boyer, H.W., and Cozzarelli, N.R. (1977) Interaction of bacteriophage T4 RNA and DNA ligases in joining of duplex DNA at base-paired ends. *J. Biol. Chem.* **252**, 3987–3994
10. Sgaramella, V. and Khorana, H.G. (1992) A further study of the T4 ligase-catalyzed joining of DNA at base-paired ends. 1972 [classical article]. *Biotechnology* **24**, 28–37
11. Ciarrocchi, G., Lestingi, M., Wright, G., and Montecucco, A. (1993) Bacteriophage T4 and human type I DNA ligases relax DNA under joining conditions. *Nucleic Acids Res.* **21**, 5934–5939
12. Tsytovich, A.V., Dolinnaia, N.G., and Shabarova, Z.A. (1988) T4-DNA ligase: substrate properties of synthetic DNA-duplexes with structural anomalies. *Mol. Biol. (Mosk)* **22**, 690–699
13. Harada, K. and Orgel, L.E. (1993) Unexpected substrate speci-

ficity of T4 DNA ligase revealed by in vitro selection. *Nucleic Acids Res.* **21**, 2287–2291
14. Pritchard, C.E. and Southern, E.M. (1997) Effects of base mismatches on joining of short oligodeoxynucleotides by DNA ligases. *Nucleic Acids Res.* **25**, 3403–3407
15. Wiaderkiewicz, R. and Ruiz-Carrillo, A. (1987) Mismatch and blunt to protruding-end joining by DNA ligases. *Nucleic Acids Res.* **15**, 7831–7848
16. Wu, D.Y. and Wallace, R.B. (1989) Specificity of the nick-closing activity of bacteriophage T4 DNA ligase. *Gene* **76**, 245–254
17. Gupta, N.K., Ohtsuka, E., Weber, H., Chang, S.H., and Khorana, H.G. (1968) Studies on polynucleotides. LXXXVII. The joining of short deoxyribopolynucleotides by DNA-joining enzymes. *Proc. Natl. Acad. Sci. USA* **60**, 285–292
18. Pyshnyi, D.V., Krivenko, A.A., Lokhov, S.G., Ivanova, E.M., Dymshits, G.M., and Zarytova, V.F. (1998) Interaction of short oligonucleotides derivatives with nucleic acids. VI. Discrimination of mismatch-containing complexes upon ligation of a short oligonucleotide tandem on DNA template. *Bioorg. Khim.* **24**, 32–37
19. Shilov, I.A., Koroleva, O.N., and Drutsa, V.L. (1993) Features of connecting short oligonucleotides with phage T4 DNA ligase. *Mol. Biol. (Mosk)* **27**, 647–654
20. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Doherty, A.J. and Dafforn, T.R. (2000) Nick recognition by DNA ligases. *J. Mol. Biol.* **296**, 43–56
22. Allawi, H.T. and SantaLucia, J., Jr. (1998) NMR solution structure of a DNA dodecamer containing single G"T mismatches. *Nucleic Acids Res.* **26**, 4925–4934
23. Allawi, H.T. and SantaLucia, J., Jr. (1997) Thermodynamics and NMR of internal G·T mismatches in DNA. *Biochemistry* **36**, 10581–10594
24. Allawi, H.T. and SantaLucia, J., Jr. (1998) Nearest neighbor thermodynamic parameters for internal G·A mismatches in DNA. *Biochemistry* **37**, 2170–2179
25. Peyret, N., Seneviratne, P.A., Allawi, H.T., and SantaLucia, J., Jr. (1999) Nearest-neighbor thermodynamics and NMR of DNA sequences with internal A·A, C·C, G·G, and T·T mismatches. *Biochemistry* **38**, 3468–3477
26. Allawi, H.T. and SantaLucia, J., Jr. (1998) Nearest-neighbor thermodynamics of internal A·C mismatches in DNA: sequence dependence and pH effects. *Biochemistry* **37**, 9435–9444
27. Allawi, H.T. and SantaLucia, J., Jr. (1998) Thermodynamics of internal C·T mismatches in DNA. *Nucleic Acids Res.* **26**, 2694–2701
28. Harvey, C.L. and Wright, R. (1972) Ligase joining of oligodeoxythymidylates. *Biochemistry* **11**, 2667–2671
29. Harvey, C.L., Gabriel, T.F., Wilt, E.M., and Richardson, C.C. (1971) Enzymatic breakage and joining of deoxyribonucleic acid. IX. Synthesis and properties of the deoxyribonucleic acid adenylate in the phage T4 ligase reaction. *J. Biol. Chem.* **246**, 4523–4530
30. Rossi, R., Montecucco, A., Ciarrocchi, G., and Biamonti, G. (1997) Functional characterization of the T4 DNA ligase: a new insight into the mechanism of action. *Nucleic Acids Res.* **25**, 2106–2113