Volume 15 Number 21 1987 Nucleic Acids Research

Nicks 3′ or 5′ to AP sites or to mispaired bases, and one-nucleotide gaps can be sealed by T4 DNA ligase

Colette Goffin, Véronique Bailly and Walter G.Verly

Laboratoire de Biochimie, Faculté des Sciences, Université de Liège, Sart Tilman B6, 4000 Liège I, Belgium

Received July 22, 1987; Revised and Accepted October 1, 1987

Summary : Using synthetic oligodeoxynucleotides with 3'-OH ends and ^{32}P-labelled 5'-phosphate ends and the technique of polyacrylamide gel electrophoresis, it is shown that, in the presence of the complementary polynucleotide, an AP (apurinic or apyrimidinic) site at the 3' or the 5' end of the labelled oligodeoxynucleotides does not prevent their ligation by T4 DNA ligase, although the reaction rate is decreased. This decrease is more severe when the AP site is at the 3' end; the activated intermediates accumulate showing that it is the efficiency of the adenyl-5'-phosphate attack by the 3'-OH of the base-free deoxyribose which is mostly perturbed.

Using the same technique, it is shown that a mispaired base at the 3' or 5' end of oligodeoxynucleotides does not prevent their ligation. A one-nucleotide gap, limited by 3'-OH and 5'-phosphate, can also be closed by T4 DNA ligase although with difficulty; here again the activation of the 5'-phosphate end does not seem to be slowed down, but rather the 3'-OH attack of the adenyl-5'-phosphate.

All these anomalous ligations take place with the nick or the gap in front of a continuous complementary strand. Blunt ends ligation of correct duplexes occurs readily; however an AP site or a mispaired base at the 3' or 5' end of one strand of the duplexes prevents ligation between these strands. But a missing nucleotide (responsible for one unpaired nucleotide protruding at the 3' or 5' end of the complementary strand) does not stop ligation of the shorter oligodeoxynucleotides between independent duplexes.

INTRODUCTION

We have shown a few years ago [1] and recently confirmed [2] that T4 DNA ligase can seal the nick produced by AP endonucleases devoid of exonuclease activity (such as the chromatin AP endonuclease of rat liver, or E.coli endonuclease IV) 5' to AP sites in circular ϕX174 phage RF DNA or in linear T7 phage DNA; the ligation takes place in spite of the absence of a base on the 5'-phosphorylated deoxyribose end. The AP site is present in the reconstituted continuous strand as can be shown by a second treatment with the AP endonuclease or by an alkaline treatment.

The same demonstration can be done with synthetic polynucleotides. In this paper, we show that oligonucleotides

© IRL Press Limited, Oxford, England.

pd(-)C(TC)$_4$, with a phosphorylated base-free deoxyribose at the 5' end, when hybridized to polyd(AG) are readily ligated together by T4 DNA ligase. We are also interested in the action of T4 DNA ligase on nicks with 3'-OH and 5'-phosphate ends 3' to AP sites. It is likely only an academic question since we know of no enzyme which has been shown to hydrolyze the phosphodiester bond 3' to an AP site; the so-called "3' AP endonucleases" (or AP endonucleases class I [3]) are β-elimination catalysts ([4] and unpublished results) and, if the break produced by β-elimination is limited by a 5'-phosphate on one side, there is no 3'-OH on the other side but rather a 2'-3' double bond; although it was obvious that T4 DNA ligase could not seal such a break, we confirmed it experimentally (not shown). Because of that, we could study our academic question only with a synthetic substrate. We show here that oligonucleotides pd(CT)$_4$C(-), with a base-free deoxyribose at the 3' end, when hybridized to polyd(AG), can be sealed by T4 DNA ligase. The ligation is however slow and the adenyl-5'-phosphate activated intermediates accumulate showing that it is the final ligation step which is retarded.

A mispaired base on the 3' or 5' side of a nick limited by 3'-OH and 5'-phosphate does not prevent ligation. Even the absence of one nucleotide does not forestall ligation across a gap limited by 3'-OH and 5'-phosphate ends.

MATERIALS and METHODS

Enzymes :

Calf-intestine alkaline phosphatase was bought from Sigma; T4 polynucleotide 5'-OH kinase from Pharmacia; T4 DNA ligase from Bethesda Research Laboratories. Snake venom phosphodiesterase (Sigma) was purified on a Blue-Sepharose column to eliminate the contaminating phosphatase [5].

Polyacrylamide gel electrophoresis :

The oligonucleotide solution is evaporated to dryness by vacuum centrifugation and the residue dissolved in 10 µl of stop solution (90 % formamide; 100 mM Tris.borate, pH 8.3; 2 mM EDTA; 0.05 % xylene cyanol; 0.05 % bromophenol blue). The samples are deposited in the wells (10 mm) of a vertical slab

(0.8 x 330 x 400 mm) of denaturing polyacrylamide gel (19 % acrylamide; 1 % bisacrylamide; 8.3 M urea; 100 mM Tris.borate, pH 8.3; 2 mM EDTA). The electrophoresis is carried out under a constant 700 volts; the duration depends on the length of the oligonucleotide and the resolution to be obtained, bromophenol blue which migrates with the octanucleotides being used as an indicator.

Kodak or Fuji X-ray films are used for the autoradiographies which are carried out, with a Kodak X-OMATIC superfast intensifying screen, at -70°C for about 20 h for the analytical gels, or about 5 min at room temperature for the preparative gels.

Oligonucleotides with 5'-OH and 3'-OH ends :

$d(T)_8$, $d(T)_8A$, and $d(CT)_4$: 8.3 nmol $pd(T)_8$, $pd(T)_8A$, or $pd(CT)_4$ (Pharmacia) was incubated with 1 unit of alkaline phosphatase in 50 µl 50 mM Tris.HCl, pH 8.6, 1 mM EDTA, 64 mM $(NH_4)_2SO_4$ for 1 h at 37°C. The enzyme was inactivated by a 10-min heating at 70°C or by chloroform extraction.

$d(CT)_5$, $d(CT)_4C$, $d(CT)_4CA$, $dAC(TC)_4$, and $d(AG)_{20}$: were made on an automated DNA synthesizer (3810 Bio-Research) using the phosphoramidite method. The oligonucleotide chain with the protective groups, attached to the silica support, was treated 5 h at 50°C in 2 M ammonium hydroxide. After centrifugation, the supernatant was chromatographed in 50 mM ammonium bicarbonate on Sephadex (G-50 for $d(AG)_{20}$; G-10 for the others). After lyophilization, the oligonucleotides were dissolved in 0.1 mM EDTA.NaOH, pH 8.0 (1 µg/µl) and kept at -20°C.

5'-Phosphate labelled $pd(T)_8$, $pd(T)_8A$, $pd(CT)_4$, $pd(CT)_5$, $pd(CT)_4C$, $pd(CT)_4CA$, and $pdAC(TC)_4$: 8.3 nmol of the oligonucleotide was incubated with 10 units of polynucleotide 5'-OH kinase, 42 nmol ATP and 120 µCi [γ-^{32}P]ATP (3 Ci/µmol;Amersham) in 50 µl 60 mM Tris.HCl, pH 8.0, 9 mM $MgCl_2$, 100 mM KCl, 15 mM 2-mercaptoethanol, for 1 h at 37°C. The reaction was stopped with EDTA.NaOH, pH 8.0, to reach 20 mM final concentration. The 5'-[^{32}P]phosphate labelled oligonucleotide was purified by electrophoresis on denaturing polyacrylamide gel. After autoradiography, the correct band was excised and eluted with 0.1 mM EDTA.NaOH, pH 8.0. The eluate was chromatographed on a NENSORB

column (NEN Research Products) : after washing with 100 mM Tris. HCl, pH 7.7, 1 mM EDTA, 10 mM triethylamine, a second washing with water was carried out before eluting the oligonucleotide with 20 % ethanol in water (v:v). After evaporation of the solution, the oligonucleotide was dissolved in 0.1 mM EDTA.NaOH, pH 8.0, at a concentration of 0.2 µg/µl, and kept at 4°C. The specific radioactivity was 6.5 x 10^6 dpm/nmol.

5'-Phosphate labelled $pd(T)_8(-)$ and $pd(CT)_4C(-)$: 0.3 nmol of 5'-phosphate labelled $pd(T)_8A$ or $pd(CT)_4CA$ was depurinated 1 h at 65°C in 10 mM HCl; the solution was neutralized by addition of 20 mM NaOH. The 3'-terminal base-free deoxyribose is indicated by (-).

5'-Phosphate labelled $pd(-)C(TC)_4$: when 5'-phosphate labelled $pdAC(TC)_4$ was depurinated in the same conditions as $pd(CT)_4CA$, after electrophoresis, most of the radioactivity was found in a doublet at the level of a pentose-phosphate; this indicates that the depurination was quickly followed by a β-elimination which cleaved the phosphodiester bond 3' to the AP site [4]. We rather followed one of the methods used by Tamm et al.[6] to prepare high-molecular weight apurinic acid : 0.3 nmol of the 5'-phosphate labelled $pdAC(TC)_4$ was incubated 24 h at 37°C in 30 mM HCl, 0.1 mM EDTA; the solution was subsequently neutralized by addition of 1/2 volume 60 mM NaOH, 2.4 mM EDTA, 50 mM Hepes. The 5'-terminal base-free deoxyribose is indicated by (-).

Treatment with T4 DNA ligase :

The 5'-phosphate labelled oligonucleotide (0.035 nmol), in the presence of the complementary polynucleotide, is incubated with 2.8 or 4.7 units of T4 DNA ligase in 20 µl 50 mM Hepes.KOH, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 µM ATP, 0.1 mM EDTA. As complementary polynucleotide, $pd(A)_{40-60}$ (Pharmacia) is used in the experiments with the homo-polynucleotides $pd(T)_8$, $pd(T)_8A$, $pd(T)_8(-)$; $d(AG)_{20}$ is used with the alternate oligonucleotides $pd(CT)_4$, $pd(CT)_5$, $pd(CT)_4C$, $pd(CT)_4CA$, $pd(CT)_4C(-)$, $pdAC(TC)_4$, $pd(-)C(TC)_4$. Details are given in the legends of the figures.

Treatment with snake venom phosphodiesterase :

An aliquot of the solution, containing 10 pmol of the

oligonucleotides that were submitted to the T4 DNA ligase, is incubated 15 min at 10°C with 10 μunits of the phosphodiesterase. The reaction is stopped by addition of EDTA to reach 20 mM.

Reduction of base-free deoxyribose :

This treatment is performed to prevent interaction between the aldehyde group of the base-free deoxyribose and the gel during electrophoresis which could lead to breaks near AP sites responsible of band smearing.

To 4 volumes of the ligation medium containing 66 pmol of base-free deoxyribose, is added 1 volume of 2 M $NaBH_4$ and the mixture is incubated 30 min at 25°C. The $NaBH_4$ excess is destroyed with 1 volume 0.3 M Na acetate, pH 5.2. After 15 min, 94 volumes 0.1 mM EDTA.NaOH, pH 8.0, are added; 10-volume aliquots are taken, evaporated to dryness and analyzed by gel electrophoresis.

NaOH treatment :

A mixture of 4 volumes of the ligation medium and 1 volume 1 M NaOH is incubated 15 min at 37°C. This treatment produces a break near each AP site by β-elimination, followed by a partial δ-elimination. The solution is neutralized by addition of 1 volume 1 M HCl. After completing with 94 volumes of 0.1 mM EDTA.NaOH, pH 8.0, 10-volume aliquots are taken, evaporated to dryness and analyzed by gel electrophoresis.

EXPERIMENTS and RESULTS

A. - Nicks 5' to AP sites or mispaired bases :

When 5'-phosphate labelled $pd(CT)_5$, hybridized to $d(AG)_{20}$, was treated with T4 DNA ligase and the result analyzed by electrophoresis, the autoradiography showed that it was efficiently ligated into high molecular weight polymers (figure 1, lanes 11 and 12). No ligation occurred when $d(AG)_{20}$ was omitted (not shown) indicating that the T4 DNA ligase preparation did not contain RNA ligase activity.

After 2 h (lane 11), $pd(CT)_5$ had completely disappeared and high molecular weight multimers were visible (up to 50-mer when examined on a 6 % polyacrylamide gel). The bands corresponding to the tetramers and multiples of tetramers are more prominent; the length of $d(AG)_{20}$ is four-times the length of

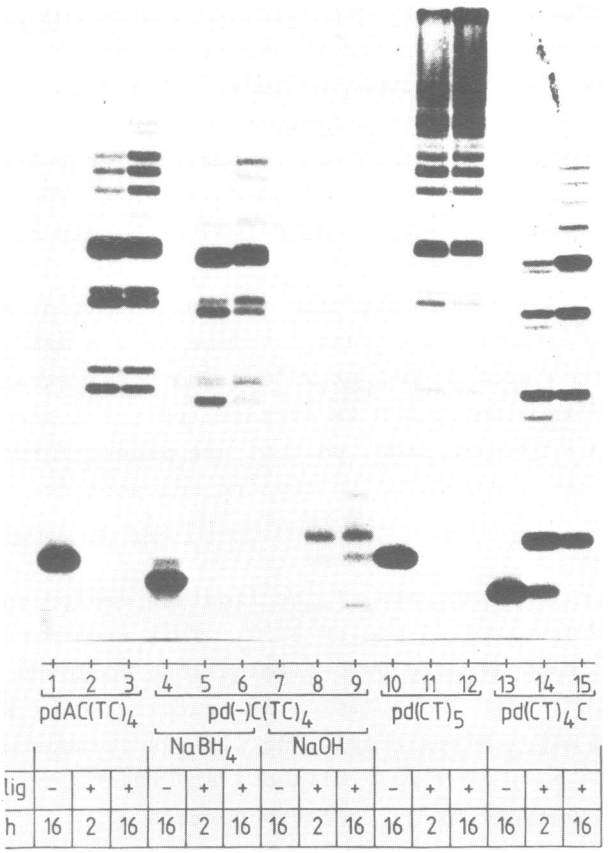

Figure 1 : Effect of a mismatch or an AP site at the 5' end on ligation of otherwise alternate co-oligonucleotides. Effect of a one-nucleotide gap on ligation.

0.35 nmol (3.5 neq nucleotides) 5'-phosphate ^{32}P-labelled $pd(CT)_5$ (lanes 10,11,12), $pdAC(TC)_4$ (lanes 1,2,3), or $pd(-)C(TC)_4$ (lanes 4 to 9), and 0.114 nmol $d(AG)_{20}$ (4.2 neq nucleotides) were incubated 5 min at 37°C in 20 mM NaCl, 0.8 mM EDTA, 15 mM Hepes.KOH, pH 7.6, then slowly cooled down to 10°C.

0.035 nmol of the hybridized radioactive oligonucleotide (230,000 dpm) was incubated at 20°C without enzyme for 16 h (lanes 1,4,7,10), or with 2.8 units of T4 DNA ligase for 2 h (lanes 2,5,8,11) or 16 h (lanes 3,6,9,12). The reaction was stopped by addition of EDTA.

For the assays containing $pd(CT)_5$ or $pdAC(TC)_4$, an aliquot of the diluted sample was dried and the residue dissolved in stop solution; a part (3,000 dpm) was then analyzed on denaturing 20% polyacrylamide gel and autoradiographed.

Each of the samples containing $pd(-)C(TC)_4$ incubated with or without T4 DNA ligase was divided in two parts : one was reduced with $NaBH_4$ (lanes 4,5,6) and the other was treated with NaOH (lanes 7,8,9) before being processed as above.

5'-Phosphate ^{32}P-labelled pd(CT)$_4$C (lanes 13,14,15) was hybridized in the same conditions and incubated at 20°C without enzyme for 16 h (lane 13), or with T4 DNA ligase for 2 h (lane 14) or 16 h (lane 15). Analysis was carried out as for pd(CT)$_5$ and pdAC(TC)$_4$.

pd(CT)$_5$, so that these prominent bands must result from a slower ligation at blunt ends of double-helices as compared to ligation in front of a continuous opposite strand. The rungs immediately above the di-, tri- and tetramers, correspond to activated intermediates as will be shown in part B with pd(T)$_8$(-).

The same experiment was carried out with 5'-phosphate labelled pdAC(TC)$_4$ and pd(-)C(TC)$_4$, also hybridized to d(AG)$_{20}$.

Figure 1, lanes 2 and 3, shows that, in spite of an A:A mispairing at the 5'-phosphate end, pdAC(TC)$_4$ was efficiently ligated up to tetramers; after 2 h (lane 2), the starting monomer had disappeared. Ligation of the tetramers to higher polymers was very slow. Thus a mispairing of the 5' nucleotide is of little consequence for ligation when it occurs in front of a continuous opposite strand, but it prevents ligation at the ends of double-helices.

The results of the experiments carried out with pd(-)C(TC)$_4$ are presented in figure 1, lanes 4 to 9. When this substrate was incubated without enzyme for 16 h at 20°C, then reduced with $NaBH_4$ before being analyzed by electrophoresis (lane 4), there was, above the major band of pd(-)C(TC)$_4$, a minor one of pdAC(TC)$_4$ which subsisted after depurination. When the incubation was made in the presence of T4 DNA ligase and the incubation products reduced by $NaBH_4$ before electrophoresis, the results were the same as with pdAC(TC)$_4$: ligation led to tetramers and not much further (lanes 5 and 6). The conclusions are thus the same. Because of the lack of one base per 10 nucleotides, the various radioactive bands are found slightly lower than when pdAC(TC)$_4$ was used as starting substrate.

To show that the [pd(-)C(TC)$_4$]$_n$ contained internal AP sites, they were treated with NaOH (compare lanes 5 and 8; 6 and 9). The multimers disappeared and products of smaller sizes are observed. In spite of the smearing due to unreduced aldehyde groups, a doublet, product of β-elimination, can be seen at the

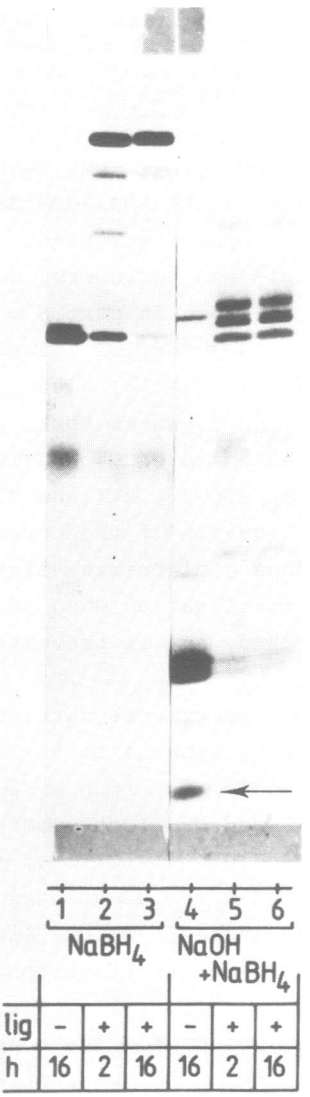

Figure 2 : Action of NaOH on the ligated $[pd(-)C(TC)_4]_n$

The experiment has been described in the legend of figure 1 concerning $pd(-)C(TC)_4$. Lanes 1, 2 and 3 correspond to lanes 4, 5 and 6 of figure 1 : the ligation products have been reduced with $NaBH_4$. Lanes 4, 5 and 6 correspond to lanes 7, 8 and 9 of figure 1 : the ligation products have been treated with NaOH; an additional reduction with $NaBH_4$ has been performed to avoid smearing of the radioactive bands. The difference was in the duration of the electrophoresis : it was stopped when the bromophenol blue had migrated 14 cm instead of 26 cm; this allows to see, especially in lane 4, the radioactive doublet of the sugar-phosphate and, below, the band of inorganic phosphate (←).

level of the decanucleotides; the other radioactive band which is situated below was the result of βδ-elimination.

To demonstrate that the AP site was really at the 5'end of the oligonucleotide, pd(-)C(TC)$_4$ was treated with NaOH, then reduced with $NaBH_4$ and analyzed by electrophoresis; the duration of electrophoresis was decreased so that inorganic phosphate did not leave the gel. Figure 2, lane 4, shows that such a treatment led to the formation of a doublet of radioactive sugar-phosphate (β-elimination) and radioactive inorganic phosphate (βδ-elimination). When the multimers resulting from the ligation of pd(-)C(TC)$_4$ were submitted to the same treatment and the same analysis (lanes 5 and 6), the yield of radioactive sugar-phosphate and inorganic phosphate was much reduced; indeed, only the 5' AP site could contribute to the formation of these radioactive species, the internal AP sites gave fragments forming three rungs (β-elimination doublet and βδ-elimination band) in the region of decanucleotides.

We can thus conclude that, with the synthetic oligonucleotide, we reproduce the result already obtained with depurinated ΦX174 phage RF DNA and T7 phage DNA treated with the chromatin AP endonuclease of rat liver [1] : a 5'-phosphorylated base-free deoxyribose limiting a nick which has a 3'-OH on the other side, does not prevent T4 DNA ligase from closing the nick. By contrast, a 5'-terminal base-free deoxyribose considerably inhibits ligation at the end of double-helices.

B. - Nicks 3' to AP sites or mispaired bases :

B.1. - With homo-polynucleotides : When 5'-phosphate labelled pd(T)$_8$, hybridized to pd(A)$_{40-60}$, was treated with T4 DNA ligase and the result analyzed by electrophoresis, the autoradiography showed that it was efficiently ligated into high molecular weight polymers (figure 3, lanes 8 and 9).

The same experiment was carried out with 5'-phosphate labelled pd(T)$_8$A and pd(T)$_8$(-) also hybridized to pd(A)$_{40-60}$.

Figure 3, lane 2, shows that, with pd(T)$_8$A, after 2 h with T4 DNA ligase, a new prominent band had appeared which migrated slightly slower than pd(T)$_8$A, and a faint one corresponding to the dimer [pd(T)$_8$A]$_2$. After 16 h (lane 3), the pd(T)$_8$A band had nearly disappeared, the intensity of the rung immediately above

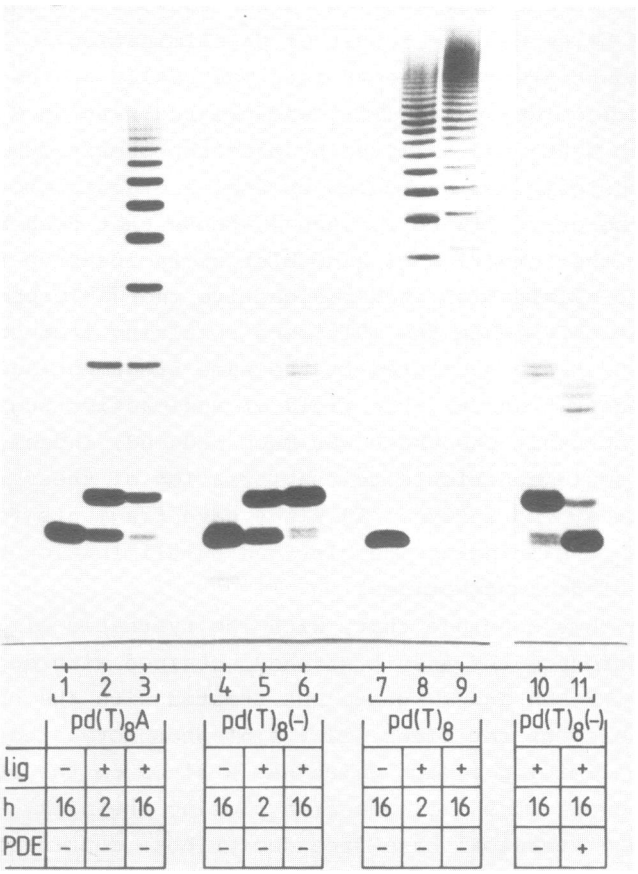

Figure 3 : Effect of a mismatch or an AP site at the 3' end on ligation of otherwise homo-oligonucleotides.

0.033 nmol (230,000 dpm) 5'-phosphate ^{32}P-labelled pd(T)$_8$ (0.264 neq nucleotides)(lanes 7,8,9), pd(T)$_8$A (0.297 neq nucleotides)(lanes 1,2,3), or pd(T)$_8$(-) (lanes 4,5,6) were hybridized to pd(A)$_{40-60}$ (0.300 neq nucleotides) and incubated at 15°C without enzyme for 16 h (lanes 1,4,7), or with 4.7 units T4 DNA ligase for 2 h (lanes 2,5,8) or 16 h (lanes 3,6,9) in a total volume of 20 µl. For lanes 10 and 11, the pd(T)$_8$(-) substrate was first treated with T4 DNA ligase as the sample analyzed in lane 6, then, after heat inactivation of the ligase, snake venom phosphodiesterase (10 µunits) was added (lane 11) or not (lane 10), and the solutions incubated 15 min at 10°C. In all cases, after stopping the reaction with EDTA and dilution, an aliquot was dried and dissolved in stop solution; a part (3,000 dpm) was then analyzed on denaturing 20 % polyacrylamide gel which was autoradiographed.

had decreased, and bands corresponding to oligomers of increasing sizes (dimers, trimers, tetramers, etc.) are clearly visible. It is concluded that a mispaired base at the 3' end

of the oligonucleotide does not prevent ligation by the T4 enzyme. As we shall see below, the rung immediately above $pd(T)_8A$ is the adenyl-5'-phosphate derivative, showing that it is the reaction between the activated 5'-phosphate and the 3'-OH of the mispaired nucleotide which is slowed down.

Figure 3, lanes 5 and 6, shows that $pd(T)_8(-)$ did not polymerize into higher molecular weight compounds in the presence of T4 DNA ligase (the faint bands in the position of dimers are likely due to the small amount of $pd(T)_8A$ that remained in the preparation). The only new band that appeared is slightly above $pd(T)_8(-)$; after 16 h, this new band had nearly completely replaced $pd(T)_8(-)$. If, after 16 h with T4 DNA ligase, this enzyme was inactivated before treating with snake venom phosphodiesterase which has a nucleotide pyrophosphatase activity [5], the new band was almost absent from the autoradiogram and replaced by a band of $pd(T)_8(-)$ (lane 11). This proves that the new band was the oligonucleotide activated at its 5'-phosphate end : the pyrophosphatase activity hydrolyzed away the adenylate and the 3'-5' exonuclease activity was blocked by the base-free deoxyribose so that the product of the reaction was the starting substrate. Thus the presence of a 3'-terminal base-free deoxyribose allowed the activation of the 5' end, but prevented the ligation step.

In the preceding experiment with $pd(T)_8A$, the rung, in the autoradiogram, immediately above the substrate is undoubtedly also the activated intermediate. We could however not prove it in the same way because, after removal of the adenylate, the phosphodiesterase degrades the oligonucleotide.

B.2. - <u>With alternate co-polynucleotides</u> : when ligation of 5'-phosphate labelled $pd(CT)_4$ was carried out, using a $d(AG)_{20}$ template/$pd(CT)_4$ nucleotide ratio of 1.3, polymers of very high molecular weights, mostly multiples of 40 nucleotides, were obtained showing a quick assembly up to pentamers, followed by a slower end to end ligation of these pentamers which had the length of the $d(AG)_{20}$ template (not shown). Results easier to compare with the experiments using other alternate co-polynucleotides were obtained with a $d(AG)_{20}/pd(CT)_4$ ratio of 13 (figure 4, lanes 11 and 12). After 2 h with the enzyme, ligation pro-

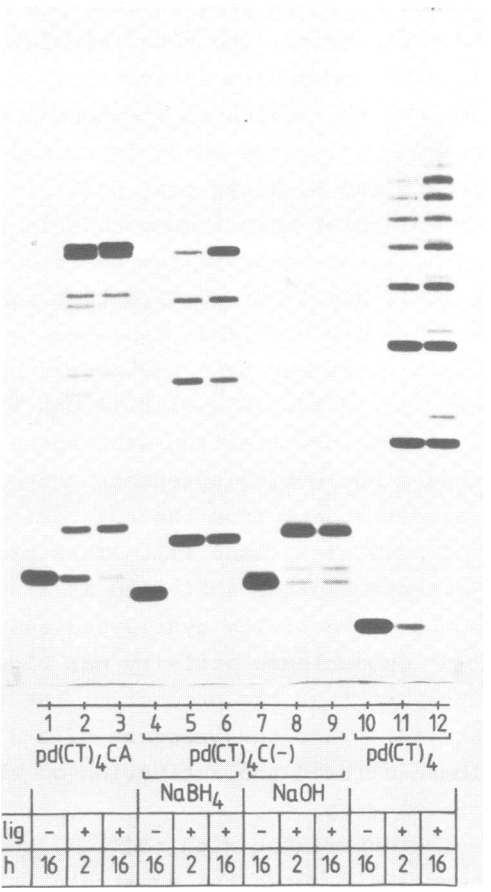

Figure 4 : Effect of a mismatch or an AP site at the 3' end on ligation of otherwise alternate co-oligonucleotides.

0.033 nmol (230,000 dpm) 5'-phosphate ^{32}P-labelled pd(CT)$_4$ (0.264 neq nucleotides)(lanes 10,11,12), pd(CT)$_4$CA (0.330 neq nucleotides)(lanes 1,2,3), or pd(CT)$_4$C(-) (lanes 4 to 9), were incubated 3 min at 45°C together with d(AG)$_{20}$. The nucleotide ratio was 1.3 for d(AG)$_{20}$/pd(CT)$_4$CA or pd(CT)4C(-), and 13 for d(AG)$_{20}$/pd(CT)$_4$. The tubes were slowly cooled down to 10°C. The hybridized radioactive oligonucleotides were incubated at 20°C (except for pd(CT)$_4$ where the temperature was 30°C) without enzyme for 16 h (lanes 1,4,7,10), or with 2.8 units T4 DNA ligase for 2 h (lanes 2,5,8,11) or 16 h (lanes 3,6,9,12), in a total volume of 20 µl. The reaction was stopped by addition of EDTA.

In the assays using pd(CT)$_4$C(-), the sample was divided in two parts : one was reduced with NaBH$_4$ (lanes 4,5,6), the other was treated with NaOH (lanes 7,8,9) before analysis. In all cases, an aliquot (3,000 dpm) was evaporated to dryness and the residue dissolved in stop solution. Electrophoresis on denaturing 20 % polyacrylamide gel was followed by autoradiography.

ducts multiple of 8 nucleotides were formed (lane 11). After 16 h, the $pd(CT)_4$ substrate had disappeared and the molecular weights of the multimers had moved upwards (lane 12). Each rung for a multimer is associated with a fainter and slower band corresponding to the activated intermediate.

With 5'-phosphate labelled $pd(CT)_4CA$, after 2 h (lane 2), the strong band immediately above the substrate corresponds to the activated intermediate. Bands corresponding to dimers, trimers and tetramers can also be seen, each associated with a band of equal intensity due to the activated intermediate. The dimer band is the faintest and the reaction has stopped at the tetramer. After 16 h (lane 3), there was less substrate left, but the situation was not very different. We conclude, as we did before with the homo-polynucleotides, that mispairing at the 3' end of the oligonucleotide does not prevent activation of the 5'-phosphate end, but slows down the final ligation step. On the other hand, the polymerization cannot go further than forming a strand having the length of the $d(AG)_{20}$ template.

Experiments were also made with 5'-phosphate labelled $pd(CT)_4C(-)$. After incubation with or without T4 DNA ligase, the reaction medium was split in two : one half was reduced with $NaBH_4$ whereas the other was treated with NaOH.

Let us examine first the results after $NaBH_4$ reduction. Lane 5 shows that, after 2 h, not only was the substrate converted into the activated intermediate, but it also gave rise to dimers, trimers and tetramers. After 16 h, no initial substrate remained and most of it was incorporated into tetramers (lane 6). One can also see that, at each level of polymerization, the major band corresponds to the activated intermediate. The results are thus similar to those obtained with $pd(CT)_4CA$: the formation of the activated intermediate is as easy, but it seems that the ligation step is more difficult although not impossible. The polymerization did not go further than the tetramer which has the same length as the $d(AG)_{20}$ template; ligation at blunt ends is thus impossible.

When the polymerization products were treated with NaOH instead of $NaBH_4$ (lanes 8 and 9), dimers, trimers and tetramers disappeared indicating that they contained internal AP sites. A

doublet stands at the level of pd(CT)$_4$C(-) resulting from β-elimination at AP sites; a slight band below the doublet must be the result of some βδ-elimination. The band at the level of the adenyl-pd(CT)$_4$C(-) has increased compared to what is observed after reduction with $NaBH_4$ without NaOH treatment; the supplementary material came from the degradation of the activated dimers, trimers and tetramers; here also, there is a splitting of the band.

C. - Ligation in absence of one nucleotide :

Using d(AG)$_{20}$ as complementary strand, we tried to ligate 5'-phosphate labelled pd(CT)$_4$C; correct pairing with the template must leave a one-nucleotide gap between successive pd(CT)$_4$C. Ligation occurred although at a slower pace than with pd(CT)$_5$. After 2 h with T4 DNA ligase (figure 1, lane 14), part of the substrate was still unreacted and the bands corresponding to the activated intermediates are the most intense. This shows that, exactly as when there is an AP site at the 3' end, it is not so much the formation of the activated intermediate which is slowed down, but rather the 3'-OH attack on the adenyl-5'-phosphate end. Nevertheless, after 16 h (lane 15), multimers of high molecular weights are clearly visible.

In another experiment where d(GA)$_5$ was used instead of d(AG)$_{20}$ and the ratio d(GA)$_5$/pd(CT)$_4$C was equal to 1, the ligation went to multimers of at least 200 nucleotides (not shown).

We conclude that a gap of one nucleotide does not prevent T4 DNA ligase from sealing an interrupted polydeoxynucleotide strand. Moreover, one unpaired nucleotide protruding at the 3' or 5' end of the complementary strand (which can itself not be ligated because of the absence of a 5'-phosphate) does not prevent blunt-end type ligation of the other strand.

DISCUSSION

In this work, we used synthetic oligonucleotides to analyze ligation when there is a mispaired base or an AP site at the 3' or 5' end of the oligonucleotides to be ligated. We also studied the consequence of a one-nucleotide gap between oligonucleotides to be ligated.

Mispaired base at the end of the oligonucleotides to be ligated :

To study the effect of a mispaired base at the 5' end, pdAC(TC)$_4$ were ligated by T4 DNA ligase in the presence of d(AG)$_{20}$. The absence of terminal 5'-phosphate prevented ligation of d(AG)$_{20}$ into higher polynucleotides. Ligation of pdAC(TC)$_4$ occurred nearly as quickly as ligation of pd(CT)$_5$ up to tetramers which had the same length as the template d(AG)$_{20}$. But, whereas [pd(CT)$_5$]$_4$ could be assembled into higher polymers, it did not occur with [pdAC(TC)$_4$]$_4$ although the tetramer was adenylated. Two conclusions can be drawn : 1°) a 5' mismatch does not disturb ligation as long as the nick is in front of a continuous opposite strand; 2°) it prevents ligation at blunt ends of double-helices although the mismatched 5' end can be activated.

The effect of a mispaired base at the 3' end was studied with pd(CT)$_4$CA. In the presence of d(AG)$_{20}$, ligation by the T4 enzyme occurred up to tetramers but did not go any further although adenyl-[pd(CT)$_4$CA]$_4$ was formed. Thus 3' mismatch and 5' mismatch have the same consequences. Tsiapalis et al.[7] have also shown that a 3' mispaired base (A:C) does not prevent ligation.

AP site at the end of the oligonucleotides to be ligated :

pd(-)C(TC)$_4$ was used to study the influence of a 5' AP site. The results are exactly the same as with pdAC(TC)$_4$: in the presence of d(AG)$_{20}$, ligation went rapidly up to tetramers and not further although activated tetramers could be observed. The presence of one 5' AP site and three internal AP sites in [pd(-)C(TC)$_4$]$_4$ was shown by alkaline degradation : β-elimination at the 5' AP site yielded a doublet of sugar-phosphate, βδ-elimination at the 5' AP site yielded inorganic phosphate, additional β- and βδ-eliminations at internal AP sites yielded a doublet of pdC(TC)$_4$(=) (with a base-free modified deoxyribose at the 3' end) and pdC(TC)$_4$p. It must be recalled that Bailly and Verly [4] have shown that β-elimination at AP sites gives two 5' cleavage products.

The previous result - ligation of pd(-)C(TC)$_4$ - confirms the observation of Goffin and Verly [1] that the nick produced 5' to an AP site by an AP endonuclease is easily closed by T4 DNA ligase.

pd(CT)$_4$C(-) was used to study the influence of a 3' AP site

on ligation. Although qualitatively identical with those obtained with pd(CT)$_4$CA, the results are kinetically different : ligation goes up to tetramers and not further, but conversion to tetramers is much slower. After 2 h, whereas, in the conditions used, most of pd(CT)$_4$CA had polymerized to tetramers, pd(CT)$_4$C(-) had only been adenylated. Thus, when the 3'-OH that attacks the adenyl-5'-phosphate belongs to a base-free deoxyribose, ligation remains possible, but it is retarded.

T4 DNA ligase can however be used as a quick test of the nature of the cleavage of the phosphodiester bond 3' to an AP site. Should the nicking have left 3'-OH and 5'-phosphate ends, T4 DNA ligase would be able to close the nick. The test could be applied to prove that the so-called 3' AP endonucleases (or AP endonucleases class I [3]) are not hydrolases but β-elimination catalysts.

However, ligation failed when the experiment was done with homo-polynucleotides : pd(T)$_8$(-) with pd(A)$_{40-60}$ as template. The reaction stopped after formation of adenyl-pd(T)$_8$(-). The most likely explanation is that the sliding of the two homopolynucleotides one relative to the other made the 5' dTMP of one oligonucleotide contiguous to the 3' dTMP of the other, expelling the base-free deoxyribose-5'-phosphate from the double-helix. This would not be the case with alternate co-polynucleotides where the d(AG)$_{20}$ template maintained a correct distance between the 5' dCMP of one pd(CT)$_4$C(-) and the 3' dCMP of the next.

One-nucleotide gap between oligonucleotides to be ligated :

pd(CT)$_4$C oligonucleotides were ligated together by T4 DNA ligase on a d(AG)$_{20}$ template. The adenylated intermediate formed readily, but the following ligation step was rather slow. Nevertheless, tetramers were obtained.

To explain that a one-nucleotide gap does not prevent ligation, one must suppose that the template dAMP in front of which there is no dTMP, must be rejected outside the double-helix, allowing the necessary close approach of the 5' dCMP of one oligonucleotide and the 3' dCMP of the next for the ligation to be possible. The same deformation of one DNA strand has been postulated by Streisinger et al.[8] to explain hot spots of spontaneous frameshift mutations in long series of identical base pairs.

The preceding conclusion obliges to come back to the discussion of the ligation of pd(CT)$_4$C(-) on a d(AG)$_{20}$ template. We said that, with these alternate co-polynucleotides (in contrast with homo-polynucleotides), the template holds the correct distance between the pd(CT)$_4$C(-) oligonucleotides so that the base-free deoxyribose cannot be expelled from the double-helix and ligation can take place between the 3'-OH of the base-free deoxyribose and the 5'-phosphate of the next oligonucleotide. This representation is probably too naive. It seems possible that the base-free deoxyribose of one strand and the dAMP of the other might be simultaneously expelled from the double-helix. This could however not be an irreversible conformational change since ligation occurs. We rather see an oscillation between a compacted form with the base-free deoxyribose-5'-phosphate and the opposite dAMP outside the double-helix, and an extended form where the alignment of the base-free deoxyribose 3'-OH permits ligation. This oscillation between conformational topoisomers might explain the slowness of the final ligation step.

Acknowledgments : This work was supported by grants from the Fonds Cancérologique de la C.G.E.R. and the Fonds de la Recherche Fondamentale Collective of Belgium. C.G. is a chargé de recherches and V.B. an aspirant of the Fonds National de la Recherche Scientifique.

References

1. Goffin,C. and Verly,W.G. (1983) Nucleic Acids Res. 11, 8103-8109.
2. Bailly,V. (unpublished).
3. Mosbaugh,D.W. and Linn,St. (1980) J.Biol.Chem. 255, 11743-11752.
4. Bailly,V. and Verly,W.G. (1987) Biochem.J. 242, 565-572.
5. Oka,J., Ueda,K. and Hayaishi,O. (1978) Biochem.Biophys.Res. Comm. 80, 841-848.
6. Tamm,C., Hodes,M.E. and Chargaff,E. (1952) J.Biol.Chem. 195, 49-63.
7. Tsiapalis,G.M. and Narang,S.A. (1970) Biochem.Biophys.Res. Comm. 39, 631-636.
8. Streisinger,G., Okada,Y., Emrich,J., Newton,J., Tsugita,A., Terzaghi,E. and Inouye,M. (1966) Cold Spring Harbor Symp. Quant.Biol. 31, 77-84.