Nonenzymatic Cleavage and Ligation of DNA at a Three A·T Base Pair Site. A Two-Step "Pseudohydrolysis" of DNA

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Abstract: The chemical nuclease Mn-TMPyP/$KHSO_5$ is able to hydroxylate 5′ carbon–hydrogen bonds of deoxyribose units at three contiguous A·T base pairs sites and initiate DNA rupture. Double-strand cleavage sites consist of trinucleotide sequences with a four nucleotide 3′ stagger of the cleaved residues. Both strand nicks are identical and consist of 3′-phosphate termini facing a 5′-aldehyde residue which is easily reduced to a 5′-alcohol by $NaBH_4$. These two successive treatments (oxidation plus reduction) are equivalent to a hydrolysis of the phosphodiester bond. Such mechanism is reminiscent of DNA restriction enzymes but with 5′-OH and 3′-phosphate ends at the site of cleavage. As demonstrated on double-stranded ODN containing an $(A\cdot T)_3$ site, the chemically cleaved DNA fragments could be religated by a BrCN chemical method to re-form the starting covalently linked DNA strand. Single- and double-strand ligations were obtained with yields ranging from 30 to 85%. Integrity of the re-formed double-stranded ODNs has been controlled (i) by quantitative analysis of nucleosides released after enzymatic digestion and (ii) by using a religated ODN as a substrate for a restriction enzyme (*BglI*).

One of the main reasons nature chose phosphate diesters as linkages within DNA is that they are very stable with respect to hydrolysis in physiological conditions.[1] The enzyme-catalyzed DNA hydrolyses are due to a metal ion-assisted attack of a water molecule on the phosphate diester in order to displace a 3′-OH (case of DNase I) or a 5′-OH end (case of DNase II). Recent three-dimensional structures of endonucleases (DNAse I[2] and *Eco*RI[3]) by X-ray structure analysis provided the necessary data to understand the protein–DNA interactions at the molecular level and the origin of the efficient enzymatic DNA hydrolysis. Despite several attempts to mimic the hydrolytic activity of nucleases with transition-metal complexes, efficient chemical hydrolysis of DNA is still a challenging area.[4] All the present DNase models exhibit poor activity and cannot be used as practical tools compared to restriction enzymes.[5] The chemical nucleases which oxidatively degrade and cleave DNA leave either phosphate monoesters or modified sugar residues at the 3′ and 5′ termini. Such strand terminations are not suitable for an enzymatic DNA ligation (for recent articles on chemical nucleases, see refs 6–10). An alternative model to natural nucleases would be to hydroxylate a C–H bond at 5′, leaving a 3′-P and a 5′-aldehyde residue (cleavage event), which could be followed by a reduction of the latter end to provide a 5′-OH termination. The strand termini produced by such two-step "pseudohydrolysis" of DNA phosphodiesters might be then religated by chemical reagents. Recently we have shown that the Mn-TMPyP/$KHSO_5$ nuclease system[11] (see Figure 1 for the structure of Mn-TMPyP) is able

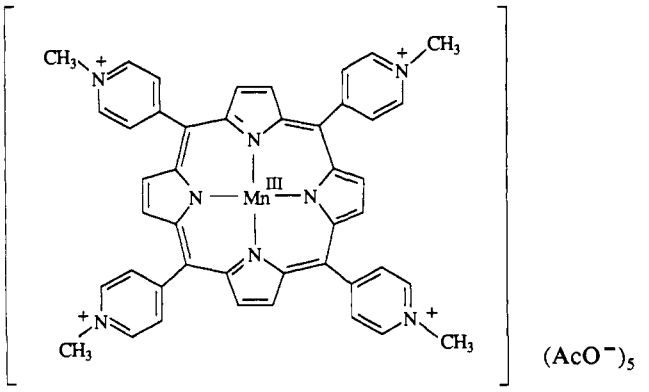

Figure 1. Structure of Mn-TMPyP, a minor groove binding molecule (the fifth ligand is probably a water molecule).

to selectively cleave DNA at A·T-rich sites (at least three consecutive A·T base pairs) by hydroxylation of one C–H bond at the 5′ position on both 3′ side deoxyriboses adjacent to the $(A\cdot T)_3$ metalloporphyrin high-affinity site (see Scheme I).[12] The activated manganese porphyrin (a putative metal–oxo species) responsible for oxidative DNA cleavage can be generated on either side of the porphyrin ring, implying that both strands of DNA can be cleaved by two single-strand breaks. This apparent double-strand lesion shows a 3′ shift, which is the signature of minor groove reacting dyes. The two DNA fragments are bearing 3′ protruding single-stranded termini overlapping on three base pairs that are reminiscent of restriction enzyme cleavage sites. The clean base pair shift is due to the strict positioning of the metalloporphyrin on its preferred binding site and to the nondiffusible high-valent porphyrin metal-oxo species. Both strand nicks are identical and consist of 3′-phosphate termini facing a 5′-aldehyde residue. By treatment with $NaBH_4$, this terminus can be readily converted to a 5′-OH end.[13] Thus the oxidative cleavage followed by a reduction step is equivalent to the hydrolysis of the phosphodiester bond (hydroxylation step + reduction step = pseudohydrolysis). In order to develop such

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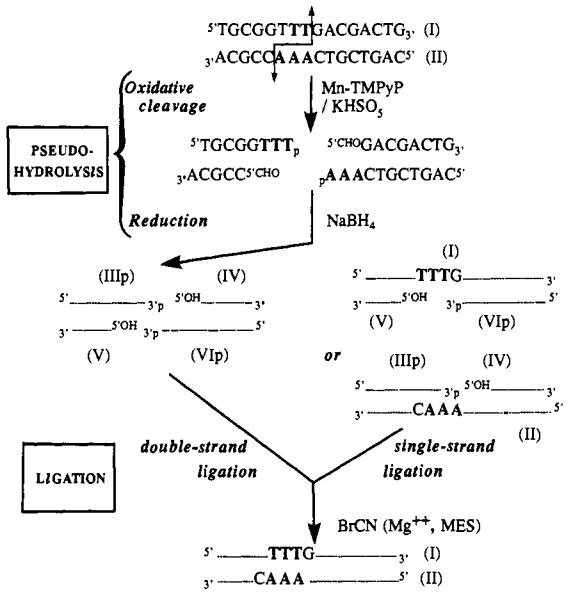
Scheme I. How To Cleave Duplex I/II (Oxidative Cleavage + Reduction = Pseudohydrolysis) and To Ligate the Resulting Fragments (Single- or Double-Strand Ligation) via Chemicals Methods



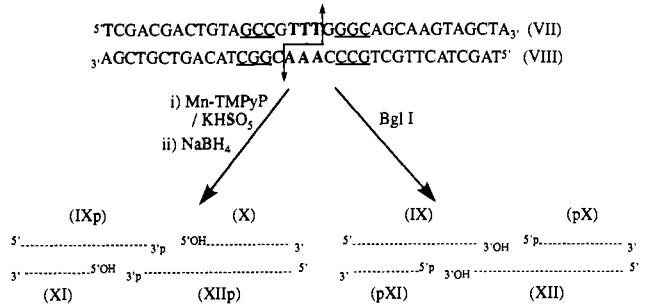
chemical tools for gene engineering, it was tempting to check if these chemically cleaved DNA fragments could be ligated to construct a covalently linked new strand of DNA. For that purpose, natural ligases could not be used because fragments to be joined were not carrying 5'-phosphate and 3'-OH ends, the usual termini, but the situation was the opposite (see above). Fortunately, chemical ligation methods described in the literature seemed especially appropriate, since the 5'-OH/3'-phosphate configuration is the most favorable for alcohol nucleophilic attack on an activated phosphate.[14-17] We chose to test the cyanogen bromide method developed by Shabarova et al.[14] In the present work this strategy has been applied to double-stranded DNA sequences containing one $(A{\cdot}T)_3$ site.

Experimental Section

Materials. Potassium monopersulfate (in fact, the triple salt $2KHSO_5{\cdot}KHSO_4{\cdot}K_2SO_4$, Curox) was a gift of Interox. Mn-TMPyP was synthesized as previously described.[10a] Cyanogen bromide was purchased from Fluka; cyclohexylamine and sodium borohydride from Aldrich. Hoechst 33342, nucleosides, *Crotalus atrox* venom phosphodiesterase I type VII, 2-morpholinoethanesulfonate (MES), and (4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonate (HEPES) were purchased from Sigma. Terminal deoxynucleotidyl transferase and [α-^{32}P]ddATP were from Boehringer. Maxam–Gilbert kit was from NEN (Dupont). *Escherichia coli* alkaline phosphatase and restriction enzyme *BglI* were from GIBCO BRL.

Preparation of Oligonucleotides. The oligodeoxyribonucleotides (ODNs) I, II, IIIp, IV, VII, and VIII[18] (see Scheme I and II) were synthesized using the β-cyanoethylphosphoramidite method on a Cyclone Plus (Milligen/Biosearch) DNA synthesizer. After cleavage of the product from the support and removal of protecting groups with ammonium hydroxide, ODNs I–IV were purified by HPLC (Nucleosil C_{18} 10-μm column) and desalted by elution through a SepPak cartridge (Millipore/Waters). ODNs VII and VIII were purified by polyacrylamide gel electrophoresis. ODN concentrations were determined by their absorbance at 260 nm using calculated extinction coefficients of 149, 154, 337.5, 327.5, 70.5, and 80 mM^{-1} cm^{-1} for I, II, VII, VIII, IIIp, and IV, respectively.[19] The 3'-phosphorylated ODN IIIp was obtained in 70% yield from the corresponding ODN carrying a 3'-terminal uridine by periodate oxidation of the 2',3'-*cis*-hydroxyl groups followed by β-elimination in the presence of cyclohexylamine.[20] All ODNs were kept dried or diluted in water as 25 μM stock solutions at 8 OD/mL for the 35-mers, 4 OD/mL for the 16-mers, or 2 OD/mL for the 8-mers (OD = optical density unit).

Scheme II. Duplex VII/VIII: Comparison of Cleavage by Mn-TMPyP/$KHSO_5$/$NaBH_4$ and *BglI* Treatment[a]



[a] Nucleotides underlined are *BglI*-recognized sequences.

Cleavage of Duplex I/II by Mn-TMPyP/$KHSO_5$. Annealing of the complementary 16-mers: 5 μL of I stock solution (0.02 OD) was mixed with 5 μL of II stock solution (0.02 OD), 5 μL of 250 mM Tris/HCl buffer, pH 8, and 5 μL of 500 mM NaCl. This saline buffer solution was heated at 90 °C for 1 min and allowed to slowly cool down to ambient temperature. The concentration of duplex was 6.25 μM. Preincubation of Mn-TMPyP with duplex: 20 μL of the above-described duplex I/II solution was allowed to equilibrate with 2.5 μL of 50 μM Mn-TMPyP diluted in water during 15 min at the temperature chosen for the following cleavage reaction. Cleavage reaction: addition of 2.5 μL of a freshly dissolved aqueous 1 mM $KHSO_5$ solution started the reaction. After 10 min at 0 °C or 4 min at 50 °C, the reaction was stopped by addition of 2.5 μL of 1 M HEPES buffer, pH 8. Further reduction of 5'-aldehyde functions on the 5' ends of fragments was carried out for 40 min at room temperature by addition of 2.5 μL of 1 M $NaBH_4$ solution. Before analysis of the cleavage products, excess $NaBH_4$ was destroyed by acetone (2.5 μL). The reaction mixture was directly analyzed by anion-exchange chromatography after having been diluted to 100 mM total salt concentration. In the case of sodium hypoiodite oxidation, 27.5 μL of 400 mM Tris/HCl buffer, pH 9, containing 2 M I_2 and 2 M NaI, was added to the cleavage reaction medium, and the reaction was kept 30 min at room temperature.[21,22] Before HPLC analysis, the sample was desalted by elution through a Sephadex G-25 superfine gel (Pharmacia) eluted with water.

Cleavage of Duplex VII/VIII by Mn-TMPyP/$KHSO_5$. ODN VII was 3' end-labeled with [α-^{32}P]ddATP with terminal nucleotidyl transferase. Annealing of the complementary ODNs VII and VIII was performed as described above. In a final volume of 50 μL of 40 mM Tris/HCl buffer, pH 8, and 100 mM NaCl, duplex VII/VIII (3'-[^{32}P]-VII, 5 × 10^4 cpm total) was 1 μM, Mn-TMPyP was 1 μM, and $KHSO_5$ was 100 μM. After 10 min of reaction in an ice bath, the cleavage reaction was stopped by adding 5 μL of 10 mM HEPES buffer, pH 8. Five aliquots were separated: a control was ethanol-precipitated before addition of $KHSO_5$; an aliquot was ethanol-precipitated immediately after HEPES addition; one was heated 30 min at 90 °C; another was subjected to $NaBH_4$ reduction for 1 h at ambient temperature; and the last one was reacted with NaOI[21,22] for 1 h at ambient temperature before ethanol precipitation. The samples were centrifuged for 15 min at 4 °C, washed with 75% ethanol, dried as pellets, redissolved in loading buffer, and electrophoresed on 20% denaturing polyacrylamide gel (migration 3 h at 1800–2000 V).

HPLC Analyses of ODNs and Fragments. Analyses were performed on an anion-exchange column (4.6 mm × 10 cm, Gen-Pak Fax, Millipore

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Waters) eluted in a gradient mode with 25 mM Tris/HCl buffer, pH 8.5, containing a linear gradient of NaCl from 100 to 500 mM over 60 min at a flow rate of 0.75 mL/min. The column was thermostated at 50 °C so that only the 16-mers were eluting as double-stranded duplexes. Detection was at 254 nm.

HPLC Analyses of Nucleosides. Analyses were done on a reverse-phase Nucleosil C_{18} 10-μm column, eluting with 0.1 M triethylammonium acetate buffer, pH 6.5, containing 5% of acetonitrile. Detection was at 254 nm; the flow rate was 1 mL/min. Retention times were 4.6, 8, 8.8, and 16 min for the four nucleosides, 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG), thymidine (dT), and 2'-deoxyadenosine (dA), respectively. Quantification was performed by comparison of peak areas against standard nucleosides areas.

Isolation of Cleavage Products. Single-stranded ODNs generated after Mn-TMPyP/$KHSO_5$ cleavage of duplex I/II were collected from a 20-fold-scale experiment chromatographed as described above. The collected DNA fragments were desalted by elution through Sephadex G25 superfine gel (10 mL of gel, elution with water) and lyophilized to dryness. Quantification was done by HPLC analysis of aliquots of aqueous solutions of fragments, in order to adjust their concentration to 25 μM stock solution. Calculated molar extinction coefficients for V and VIp are 45 and 110 $mM^{-1}\,cm^{-1}$, respectively. From the initial 20-fold-scale duplex cleavage experiment (0.4 OD of each 16-mer) one could recover 0.2 OD (25%) of all fragments together (IIIp, IV, V, VIp) after cleavage, purification, and desalting procedures.

Chemical Ligation. Chemical ligations were performed with the above isolated single-stranded ODNs. For single-stranded nicked duplexes, only the two fragments corresponding to one strand were hybridized onto the synthetic complementary 16-mer template. In the case of double-stranded nicked duplex, the four fragments of cleavage were mixed together. A mixture of 5 μL of each 25 μM stock solution of the oligomers necessary to reconstruct the duplex I/II was lyophilized, and the residue was dissolved in 9 μL of 250 mM MES/triethylamine buffer, pH 8, 20 mM $MgCl_2$. Annealing was performed as previously stated, and the solution was then kept at 0 °C for at least 24 h. The concentration of nicked duplex, the substrate for ligation, was about 14 μM. In some samples, Mn-TMPyP or Hoechst dye was added to this annealing medium at a concentration of 25 μM. Chemical ligation reaction was carried out in an ice bath. To 9 μL of the nicked duplex solution was added 0.5 μL of 1.25 M BrCN diluted just before use in water. This addition was repeated three times over 20 min. In these conditions, final concentrations were 200 mM, 11.5 μM, and 230 mM for MES, duplex, and BrCN, respectively; final pH was 6. Before HPLC analysis, excess BrCN was destroyed by addition of 15 μL of 200 mM phosphate buffer, pH 8, and the sample was diluted to a final 100 mM salt concentration.

Yield of Ligation. The ligation yield was estimated from the HPLC chromatogram obtained at the end of the ligation reaction using the following formula:

$$Y = 100 \times \frac{D/\epsilon_D}{A/\epsilon_A + D/\epsilon_D}$$

where A represents the peak area of the nicked duplex fragment present in minor amount (when the mixture was not strictly stoechiometric), D is the peak area of the formed duplex, and ϵ_A and ϵ_D are the corresponding absorption coefficients. We checked in control experiments that determination of concentrations using peak area/absorption coefficient ratios was valid in the HPLC conditions used.

Enzymatic Digestion of Religated Duplex I/II. A mixture of 40 μL each of 25 μM solution of the four fragments IIIp, IV, V, and VIp (from Mn-TMPyP/$KHSO_5$/$NaBH_4$ reaction) was lyophilized and religated according to previously described experimental procedures. The resulting duplex was collected from HPLC, desalted, and digested by venom phosphodiesterase (2.5 units) and alkaline phosphatase (0.75 units) in 25 μL of 100 mM, Tris/HCl buffer, pH 9, overnight at 37 °C. In this reaction, duplex concentration was 7 μM. Analysis of nucleosides was performed by HPLC and compared to the digestion of control duplex I/II under the same conditions.

***Bgl*I Digestion of Religated Duplex VII/VIII.** A large-scale (2 mL of reaction medium) cleavage experiment on labeled duplex VII/VIII with Mn-TMPyP/$KHSO_5$/$NaBH_4$ (3'-[^{32}P]VII total = 10^6 cpm) was undertaken to isolate the four fragments IXp, X, XI, and XIIp. The four bands were cut out under UV light from a 20% polyacrylamide electrophoresis gel. Fragment 3'-[^{32}P]X was also cut out after a 10-min autoradiography of the gel. ODNs were extracted from polyacrylamide, carefully desalted on a nick-spin column (Pharmacia), lyophylized, and redissolved together in 9 μL of 250 mM MES/Et_3N, pH 8, 20 mM $MgCl_2$ buffer. The estimated nicked duplex concentration was 15 μM. After addition of 1 μL of 300 μM Mn-TMPyP, double-strand ligation was accomplished by two successive 2-μL additions of 1.25 M aqueous BrCN. Chemical ligation was stopped by ethanol precipitation. The total reaction mixture was washed twice with 75% ethanol, dried, dissolved in loading buffer, and electrophoresed on a 20% denaturing polyacrylamide gel. Religated ODN VII was detected by autoradiography, extracted from the gel, desalted, and hybridized with ODN VIII (extracted from the same gel) in 20 μL of *Bgl*I buffer (50 mM Tris/HCl buffer, pH 8, 10 mM $MgCl_2$, 50 mM NaCl). The subsequent religated duplex 3'-[^{32}P]VII/VIII was incubated in the presence of 20 units of *Bgl*I enzyme at 37 °C 24 h (estimated duplex concentration = 1 μM). Half of the reaction medium was left at 37 °C for 2 h more, and the other half was heated at 65 °C for 20 min to inactivate *Bgl*I and treated with 1 μL of bacterial alkaline phosphatase (150 units) for 2 h at 37 °C. At the end of the reaction times, samples were ethanol-precipitated and treated as usual before electrophoresis. Parallel digestion of duplex was performed: 50 pmol of duplex VII/VIII (3'-[^{32}P]VII, 30 000 cpm) was incubated with 50 units of *Bgl*I at 37 °C for 24 h in 50 μL of adequate buffer. Half of the reaction medium was heated and subsequently incubated with 2 μL of alkaline phosphatase (300 units) for 2 h at 37 °C; the other half was left at 37 °C for 2 h more.

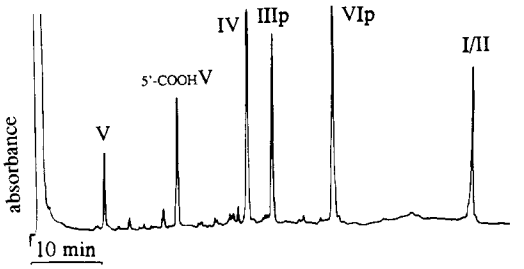

Figure 2. Anion-exchange HPLC analysis of duplex I/II cleavage by Mn-TMPyP/$KHSO_5$ followed by a reduction step. Cleavage conditions: duplex 5 μM, Mn-TMPyP 5 μM, $KHSO_5$ 100 μM, 10 min at 0 °C.

Results and Discussion

Cleavage of Duplex I/II. Cleavage of duplex I/II at 0 °C with Mn-TMPyP/$KHSO_5$ led to five single-stranded fragments easily separated on an anion-exchange column (see Figure 2). The 16-mer duplex was 80% cleaved, and the cleavage was sequence-selective. Mn-TMPyP, activated by $KHSO_5$, is able to specifically hydroxylate C–H bonds at the 5'-sugar positions on both 3' sides of the $(A\cdot T)_3$ site. This nuclease activity on both DNA strand leads to a four base pair shift in the 3' direction. One side of the nick is a 3'-phosphate end, and the other side, a 5'-aldehyde end. After reduction of the 5'-aldehyde function by $NaBH_4$, the four expected cleavage products of duplex I/II were identified on the chromatograms: IIIP, IV, V, and VIp (Scheme I). The fifth peak, not sensitive to $NaBH_4$ reduction or to heating conditions, corresponds to the 5-mer ending with a 5'-carboxylic acid sugar residue (5'-COOH) as confirmed by sodium hypoiodite oxidation of the cleavage reaction mixture.[21,22] The acid termini are probably due to a second oxidation of the cleaving system onto the 5'-CHO site. In the present case, the 5-mer was composed of 50% of alcohol-end and 50% of acid-end. No acid-ending fragments could be detected on the other strand. When the concentration of the cleaving system Mn-TMPyP/$KHSO_5$ was decreased, the respective percentages of acid *versus* alcohol were unchanged despite a significantly lower cleaving efficiency (not shown). This "non-natural" 5'-COOH terminus seems a constitutive feature of the cleaving system. It appears that the percentage of the 5'-COOH terminus is dependent on the nature of the 3' adjacent base of the affinity binding site of Mn-TMPyP, since it varies from 0–10% in the case of guanine to 50% in the case of cytosine (this work and ref 22). For ligation experiments, 5'-COOH-ending fragments should be avoided as much as possible. One way is to perform the cleavage reaction at a

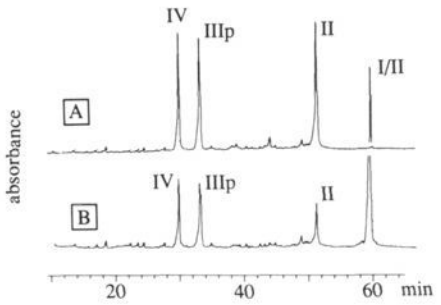

Figure 3. Anion-exchange HPLC analysis of single-strand ligation. Illustration of run 1 in Table I. (A) t_0 of the reaction; (B) end of the reaction. Yield of ligation is 75%.

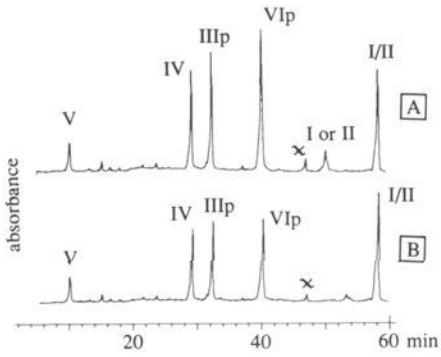

Figure 4. Anion-exchange HPLC analysis of double-strand ligation in the absence (A) or in the presence (B) of added Mn-TMPyP. Peak X is interpreted as the 13-mer V–IIIp. Figure 4 is illustrating run 4 described in Table I. Yields of ligation are 35% (A) and 45% (B).

Table I. Chemical Single- (runs 1–3) and Double- (run 4) Strand Ligation of Cleavage Fragments of Duplex I/II

	starting material[a]						ligation product I/II	
run	V	VIp	IIIp	IV	I[b]	II[b]	concn (μM)	yield (%)[c]
1			12.5	12.5		8.75	6.5	75
2	12.5	16.5			10		8.5	85
3			1.2	1.2		1	0.6	60
4	7.5	16	15	12.5			2.5–3.5	35–45

[a] Initial concentration in μM. [b] I or II template for V + VIp or IV + IIIp, respectively. [c] Calculated considering the maximum amount of duplex that can be formed from the oligomer in minor concentration.

temperature that does not allow the 5′-CHO-generated fragment to remain annealed. Increasing the cleavage reaction temperature from 0 to 50 °C showed a progressive decrease in the percentage of 5′-COOH 5-mer from 50 to 0%. At 50 °C, 60% of the duplex is cleaved (not shown), and the expected four single-stranded fragments are not in stoichiometric amounts: 60% of cleavage is observed on the A-rich strand according to previous observations.[12] Double-strand cleavage of DNA by Mn-TMPyP/$KHSO_5$ results from two independent hydroxylations of $C_{5'}$–H bonds on opposite strands and requires two consecutive oxidative activations of the metalloporphyrin by the oxygen atom donor. The reactivity of activated metalloporphyrin might be more efficient on one side due to its better position toward the target C–H bond. At 0 °C, both strands are equally cleaved, but the A-rich one is preferentially attacked compared to the T-rich strand (because of the higher proportion of acid-ending 5-mer) and the reactivity probability seems to be the same: 60% on A-side, 40% on T-side.

Religation of Duplex I/II Fragments. Two experimental tests were performed: single-strand and double-strand ligation (Scheme I, Figures 3 and 4, and Table I). The results of single-strand ligation are presented in Table I, and an example is illustrated in Figure 3. When the 16-mer template was in slightly less than a stoichiometric amount compared to the two ODNs that will be

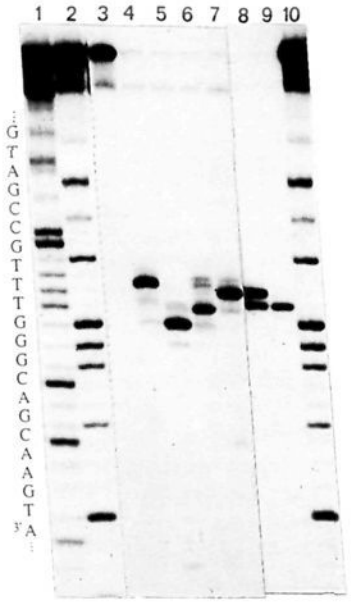

Figure 5. Polyacrylamide gel electrophoresis analysis of 35 base pair duplex VII/VIII cleavage (3′ end-labeled on strand VII). Comparison of Mn-TMPyP/$KHSO_5$ cleavage of untreated duplex (lanes 3–7) and *BglI* cleavage of cut and ligated duplex (lanes 8, 9). Lane 1: C + T Maxam–Gilbert sequencing reaction. Lanes 2 and 10: G Maxam–Gilbert sequencing reaction. Lanes 3–7: Mn-TMPyP/$KHSO_5$ cleavage analysis; lane 3, control without $KHSO_5$; lane 4, cleavage; lane 5, heating treatment; lane 6, NaOI treatment; lane 7, $NaBH_4$ treatment. Lanes 8 and 9: *BglI* cleavage of religated duplex; lane 8, *BglI* followed by alkaline phosphatase treatment; lane 9, *BglI* alone. Lanes 1, 2, 8–10 were overexposed in order to show that no starting material was left after *BglI* digestion.

ligated, ligation yields ranged from 60 to 85% (runs 1–3). Single-strand ligation experiments involving 5′-COOH-V, VIp, and I ODNs failed to give any covalently ligated II strand. Ligation of double-stranded nicked duplex, that is, the mixture of IIIp, IV, V, and VIp in approximately equal quantities, gave about 35% (based on the fragment V, which was the less concentrated one) of double-stranded covalently joined duplex as seen on Figure 4 and described in run 4 in Table I. In this latter case was observed (Figure 4A) a low percentage of ligation of only one strand (about 4% of a 16-mer, I or II; both of them coelute on HPLC) and also 1% of interstrand ligation between V and IIIp which led to a 13-mer (peak X, eluted between VIp and both 16-mers in the HPLC conditions). The lower efficiency of the chemical double-strand ligation is, of course, due to the weaker cohesion of the three A·T base pair, which maintains the entire duplex structure. In order to increase the hybridization stability of the $(A{\cdot}T)_3$ region, we performed the chemical ligation in the presence of Mn-TMPyP (two metalloporphyrins per duplex) that we used as a minor groove binder. We observed an increase of about 10% of duplex production, no trace of single-stranded 16-mer, and a lowering of interstrand ligation between V and IIIp (see Figure 4B). We also tested a classical A·T base pair specific binding drug (Hoechst 33342). While this dye promoted slightly higher yields of ligation compared to the assay without the dye, it was less efficient as a ligation enhancer than Mn-TMPyP and did not decrease the single-strand ligation nor the interstrand ligation phenomenon (not shown).

Enzymatic Digestion of Religated Duplex I/II. In order to detect any DNA damages arising from oxidative/reductive reaction or/and from high BrCN concentrations used in chemical ligations, we digested the religated duplex I/II with phosphodiesterase I and alkaline phosphatase. We obtained the expected nucleoside ratio: 9 dG/9 dC/7 dA/7dT.

Cleavage of Duplex VII/VIII. Figure 5 presents the cleavage of duplex VII/VIII (3′-[^{32}P] end-labeled strand VII) generated

by Mn-TMPyP/$KHSO_5$ system (lanes 3–7). The main cleavage fragment (the slowest moving band in lane 4) corresponds to the 5′-aldehyde-ending 16-mer. Heat-induced β-elimination provides a 5′-phosphate 15-mer that migrated similarly to the corresponding Maxam–Gilbert sequencing G band (lane 5). Sodium hypoiodite oxidation of the 5′-aldehyde-ending 16-mer gives the 5′-acid-ending 16-mer (lane 6), and $NaBH_4$ reduction leads to the 5′-OH 16-mer (lane 7). No 5′-acid-ending 16-mer was detected after Mn-TMPyP/$KHSO_5$ cleavage; this was also the case on the T-rich strand of duplex I/II. Minor bands in each lane are secondary cleavage sites revealed when relatively drastic cleavage occurred (there was no full length DNA left at the top of the gel).[12]

Religated Duplex VII/VIII is a Substrate for a Restriction Endonuclease. To check that a chemically cleaved and religated DNA shows no chemical base modifications and presents a reformed phosphodiester bond at the site of the cut, we tested whether the religated duplex could be a substrate for a restriction enzyme. We used the 35-mer duplex VII/VIII that bears a restriction site for *Bgl*I enzyme. As shown in Scheme II, Mn-TMPyP/$KHSO_5$/$NaBH_4$ and *Bgl*I cleave the same phosphodiester bonds but leave the phosphate at the site of the cut at the 3′ or the 5′ end, respectively. For *Bgl*I cleavage experiments, the two religated 35-mer strands isolated from double-strand ligation of the four cleavage fragments IXp, X, XI, and XIIp were allowed to anneal in the *Bgl*I buffer. We could thus observe a total hydrolysis of 3′ end-labeled ODN VII when incubated with *Bgl*I (Figure 5, lane 9). Furthermore, alkaline phosphatase removed the 5′-phosphate group from the *Bgl*I-generated fragment. As shown in Figure 5, the 5′-OH 16-mer fragment generated by *Bgl*I + alkaline phosphatase (lane 8) had the same electrophoretic migration as the 5′-OH 16-mer generated by the Mn-TMPyP/$KHSO_5$/$NaBH_4$ system (lane 7). In a control experiment, *Bgl*I digestion of control duplex VII/VIII showed the same cleavage pattern (not shown).

Concluding Remarks

Most of the chemical nucleases developed during the last decade cleave DNA strands through an oxidative attack on deoxyribose units. Among the observed oxidation sites, activation of C–H bonds at C-1′ or C-4′ (by bleomycin[23] or bis(*o*-phenanthroline)-copper),[6] for example) appears to be inappropriate, since such reaction is accompanied by a loss of DNA material (base, base propenal, or other deoxyribose residues) and/or leaves DNA termini at the cleavage site inadequate for the next ligation step (*i.e.*, phosphoglycolate termini in the case of DNA cleavage by bleomycin[23]). Conjugates based on iron–EDTA complexes[7] seem also to be inappropriate for a two-step pseudohydrolysis of DNA, since they produce diffusible hydroxyl radicals, creating dispersed lesions. The only hydroxylations at C-5′ or C-3′ followed by $NaBH_4$ treatment allow or should allow (case of C-3′, see ref 24 for a recent example of oxidation at C-3′) the recovery, at the cleavage site, of 5′-OH/3′-P or 3′-OH/5′-P termini, respectively, which are the required terminal functions for the further ligation step. Among the other DNA cleavers able to activate at C-5′, neocarzinostatin[25] or the enediyne cytotoxic drugs[26] should also be mentioned, but C-5′ chemistry is restricted to one strand of DNA in the case of double-strand cleavage (C-1′ or C-4′ for the other strand).

The present work shows that an alternative to chemical DNA hydrolases is an oxidative cleavage via sugar C-5′ hydroxylation followed by a reduction step, which is equivalent to the hydrolysis of a phosphodiester bond (hydroxylation step + reduction step = pseudohydrolysis step). One problem remaining for the development of the cationic manganese porphyrin as a chemical DNA restriction tool is that the $(A{\cdot}T)_3$ cleaver affinity sequence is too often encountered on random double-stranded DNA. One way to target the cleaving activity of this reagent toward a single site on DNA would be to covalently link such a cleaver with a sequence-recognition vector like an oligonucleotide.

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