Chemical Restriction: Strand Cleavage by Ammonia Treatment at 8-Oxoguanine Yields Biologically Active DNA

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Cleavage of DNA single and double strands at an 8-oxoguanine-containing nucleotide occurs in 90% yield if the modified oligonucleotide is treated with NH_3 and O_2 at 60 °C. The mechanism of this oxidative cleavage reaction was studied, and the reaction was applied to the generation of single-stranded overhangs on PCR-amplified DNA that can be ligated. As an example, the lac Z′ *gene was amplified by PCR with 8-oxoguanine modified primers, restricted by ammonia treatment, ligated into a plasmid vector, transformed in* Escherischia coli *cells, and screened for blue colonies. This method guarantees efficiencies comparable to the standard cloning procedure with restriction enzymes, and it allows the design of any 3′-overhang independent of the sequence of the cloned DNA.*

KEYWORDS:

chemical restriction · DNA cleavage · nucleotides · polymerase chain reaction

Introduction

Cloning in plasmid vectors is a useful technique for expressing DNA fragments in bacterial cells.[1] Plasmid DNA is cleaved with restriction enzymes and joined with the foreign DNA fragment. The resulting recombinant plasmid is then used to transform bacteria. In a standard procedure, the DNA fragment is amplified by PCR by using primers that include recognition sites for restriction enzymes.[2] In this manner, DNA fragments with corresponding single-stranded overhangs (sticky ends) are produced and ligated with the plasmid vector (Scheme 1 A).

An alternative strategy involves the generation of sticky ends on DNA fragments by an enzymatic strand cleavage reaction at a *modified* nucleotide, which can be incorporated into DNA fragments by PCR with modified primers.[3] This approach results in the formation of double-stranded DNA fragments modified near their 5′-ends. After enzymatic cleavage at the modifications, 3′-overhangs are obtained. We now present a cloning method that includes a *chemical* strand cleavage reaction and uses 8-oxoguanine as the modified nucleotide (Scheme 1 B).

Chemical DNA Strand Cleavage at 8-Oxoguanine

DNA strands that contain an 8-oxoguanine moiety as a modified nucleotide can be partly cleaved at the modified position with piperidine or ammonia.[4] However, the cleavage reactions described in the literature give low yields and/or were performed at high temperatures. In order to gain DNA that can be successfully ligated, the cleavage has to occur at relatively low temperatures. We used the modified DNA strand **1** and compared different basic cleavage reagents (1 M piperidine, 35% NH_3, 0.1 M Na_2CO_3, 0.1 M NaOH). We found that 35% NH_3 is by far the most efficient cleavage reagent of those tested (Scheme 2). Nevertheless, the cleavage yield after treatment with 35% NH_3 for 1 h at 60 °C was too low (33%) to be useful. The yield of the cleaved nucleotide **3** after 1 h was increased to 65% by adding O_2 to the aqueous solution, and after 14 h the cleavage occurred with 90% yield. At 90 °C a cleavage yield of 80% was already reached after 1 h, but at this higher temperature the DNA products could not be successfully ligated (see below).

The influence of O_2 clearly shows that the cleavage at the 8-oxoguanine base involves an oxidation reaction. The mechanism could be similar to the Ir(IV)-induced cleavage studied in detail by C. Burrow,[5–7] which starts with an electron transfer step and introduction of the OH group at the 5′ position of 8-oxoguanine (**1** → **4**, Scheme 3). Subsequent ring opening and

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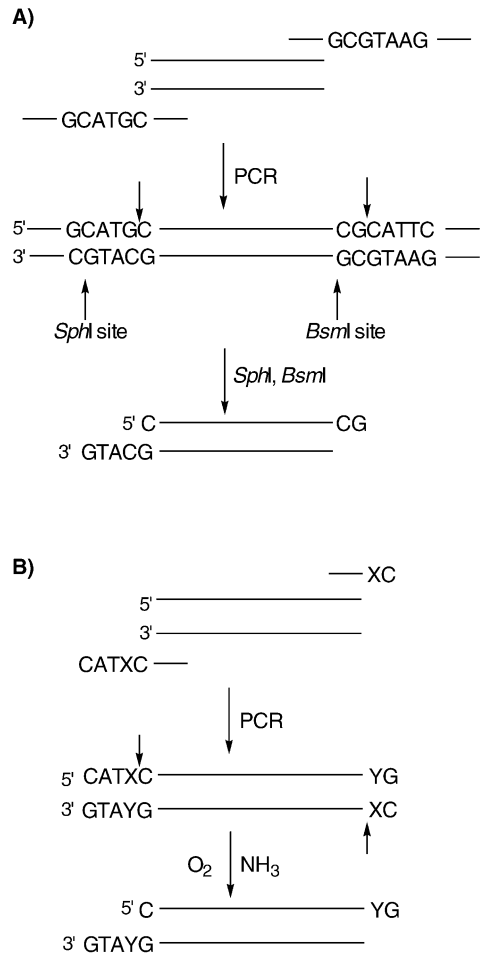
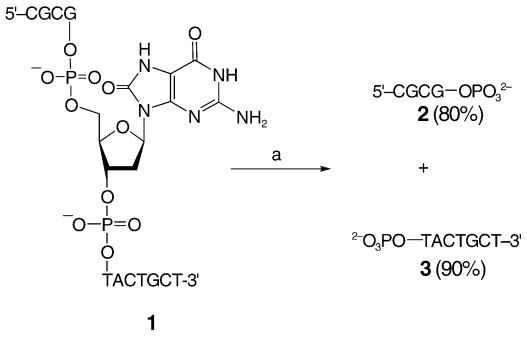
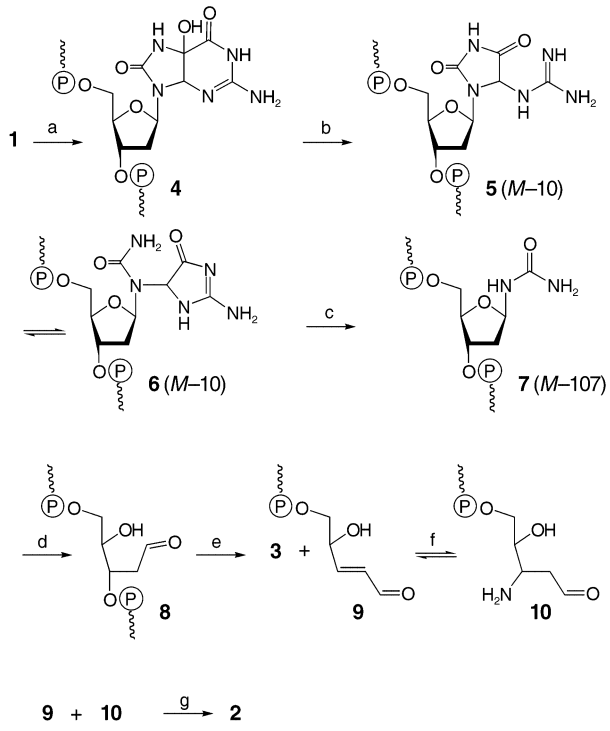


Scheme 1. *The procedure of the standard cloning method (A) starts with PCR of the DNA fragment with primers containing noncomplementary regions that include recognition sites for restriction enzymes (*SphI *and* BsmI*). Enzymatic digestion then produces sticky ends. In the alternative method (B) the PCR primers are modified with X (8-oxoguanine). Base Y (cytosine or adenine) is incorporated opposite X. Chemical restriction (ammonia, O_2) leads to the formation of single-stranded overhangs.*



Scheme 2. *Cleavage of oligonucleotide **1** at the 8-oxoguanine position. a) NH_3, O_2, H_2O; 60 °C, 14 hrs.*

decarboxylation leads to hydantoin **5**, which is in equilibrium with the amino imidazoline derivative **6**.[7] Interestingly, when we used O_2 instead of an Ir^{IV} species as the oxidant, two



Scheme 3. *Mechanism of the cleavage of oligonucleotide **1** at the 8-oxoguanine position with NH_3 and O_2. a) O_2; b) H_2O, NH_3, $-CO_2$; c) H_2O, NH_3; d – g) NH_3. M = molecular mass of **1**.*

intermediates with the same masses (MALDI-TOF) as **5** and **6** [$M-10$] were detected by HPLC.

As described for an analogous imidazolinone,[8] hydrolysis of **6** leads to the urea derivative **7**, with the mass [$M-107$], which we observed as a third intermediate. As a result of its hemiaminal-like structure, compound **7** is hydrolyzed under basic conditions to form the abasic deoxyribose **8**, which could not be observed because it undergoes rapid elimination to give the strand-cleavage products **3** and **9**. Oligonucleotide **3** is stable and was formed in up to 90% yield. In analogy to the observation of Torres,[4] α,β-unsaturated aldehyde **9** could be detected as its Michael adduct **10**. Intermediates **9** and **10**, which might be in equilibrium,[9] were cleaved under basic conditions and afforded the stable oligomer **2**. As is to be expected from this mechanism, the yield of **2** was about 10% lower than that of oligomer **3**. The time courses for the concentration of educt **1**, products **2** and **3**, and intermediate **10** were measured and are shown in Figure 1.

Additional strand scission experiments demonstrated that the cleavage reaction at 8-oxoguanine (60 °C, O_2, saturated NH_3) is not dependent on the length or the sequence of the modified oligonucleotide or on whether single or double strands are used.

Cloning with Chemical Restriction

The *lac Z′* gene on plasmid pUC19 was amplified as a 653-base DNA fragment by PCR with modified primers that contained a single 8-oxoguanosine residue. As a control, the amplification was performed with unmodified primers, which led to the same sticky ends after an enzymatic restriction (Scheme 1). The

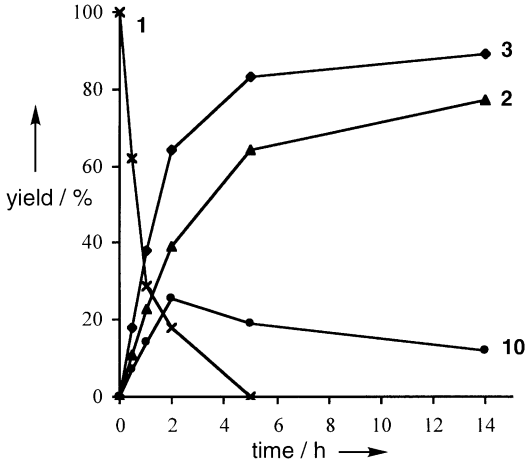


Figure 1. *Time course of the hot ammonia treatment (60 °C, O_2) of the modified (X = 8-oxoguanine) oligonucleotide 5′-CGCGXTACTGCT (**1**; crosses). Formation of 3′-phosphate **2** (triangles) and 5′-phosphate **3** (squares), as well as the concentration change of the 3′-β-amino-γ-hydroxyaldehyde **10** (circles).*

polymerases Deep Vent exo$^-$[10] and Pwo[11] were applied to both modified and unmodified primers. All four amplifications led to the formation of similar amounts of products. The modified PCR products were restricted by treatment with ammonia under various conditions, the insert DNA was ligated with the plasmid vector, and the recombinant plasmid was transformed in *Escherichia coli* cells. In the control experiment the unmodified PCR products were restricted by enzymes (*Sph*I and *Bsm*I), ligated, and transformed.

An agarose gel with restricted insert DNA and plasmid vector is shown in Figure 2. Pure DNA was obtained in similar amounts after both enzymatic and chemical restriction, and the actions of

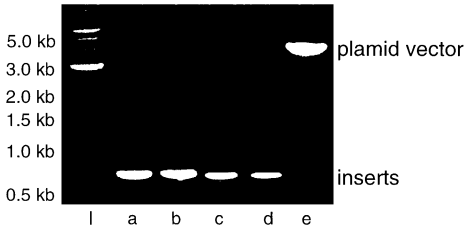


Figure 2. *Agarose gel of restricted insert DNA produced by using two thermostable polymerases: l = reference ladder; a = Deep Vent exo$^-$, unmodified primers; b = Deep Vent exo$^-$, modified primers; c = Pwo, unmodified primers; d = Pwo, modified primers; e = plasmid vector.*

both polymerases led to insert DNA bands with similar intensities. The numbers of blue colonies (colonies where the *lac Z′* gene has been successfully expressed) after ligation, transformation, and cell growth are presented in Table 1.

The efficiencies of cloning with chemical restriction by treatment with ammonia (60 °C, O_2, 14 h) were 81% (Deep Vent exo$^-$) and 85% (Pwo), respectively, relative to the standard method with restriction enzymes and unmodified DNA. The cloning yields decreased if air was used instead of O_2, and if the chemical restriction was carried out at 90 °C instead of 60 °C

Table 1. *Cloning efficiencies (blue colonies) and mutations observed opposite an 8-oxoguanine residue (*Sph*I site) when modified primers were used.*

Polymerase	Primers	Blue colonies[a]	Adenine (mutated)[b]	Cytosine (not mutated)
Deep Vent exo$^-$	modified	3370	12	1
Deep Vent exo$^-$	unmodified	4160		
Pwo	modified	4310	4	6
Pwo	unmodified	5060		

[a] Number of blue colonies obtained after chemical restriction (modified primers) by the standard method with restriction enzymes (unmodified primers), respectively. [b] Observed mutations opposite the 8-oxoguanine residue (*Sph*I site, 13 experiments with Deep Vent exo$^-$, 10 experiments with Pwo). DNA sequencing was carried out on an ABI Prism 310 analyzer after PCR amplification.

(Figure 3). If only the plasmid vector was treated with ligase, or PCR products obtained without ammonia restriction were ligated, no blue colonies were obtained.

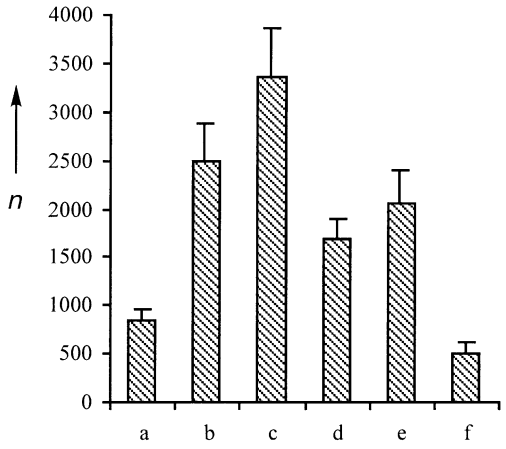


Figure 3. *Numbers of blue colonies (n) after cloning under different conditions during treatment with ammonia (Deep Vent exo$^-$): a) 60 °C, 14 h, oxygen saturation, purified by membrane filtration (Method B); b) 60 °C, 14 h, oxygen saturation, purified by ethanol precipitation (Method C); c) 60 °C, 14 h, oxygen saturation, annealed and purified by membrane filtration (Method A); d) 60 °C, 14 h, air, purification method A; e) 60 °C, 5 h, oxygen saturation, purification method A; f) 90 °C, 1 h, air, purification method A.*

In order to check the construction of the recombinant plasmids as intended, sequencing was performed across the ligation sites. All analyzed plasmids isolated from blue colonies were correctly ligated. Information on the polymerase action at the modified nucleotide was obtained by analysis of the nucleotide located opposite the 8-oxoguanine residue after PCR amplification. If cytosine was detected, the correct nucleotide had been incorporated. However, if adenine was present in the sequence, the polymerase had produced the well-described 8-oxoguanine:adenine base pair[12, 13] and, despite the mismatch in the sticky ends, a ligation must have occurred. At the *Bsm*I site, all 23 sequenced plasmids contained a cytosine residue in the relevant position. A different result was obtained at the *Sph*I site. At this site, 90% of the sequences recorded were mutated if

Deep Vent exo$^-$ was used in PCR, whereas an adenine residue was detected in 40% of the sequences if Pwo was applied (Table 1, Scheme 1).

The incorporation of adenine opposite 8-oxoguanine by polymerases has been described in several reports.[12–16] In primer extension assays, the mutation frequency was determined to be between 13% for *E. coli* DNA Polymerase I and 99% for human DNA Polymerase α.[14] In other studies, 8-oxoguanine modified plasmids were transformed in living cells. Mutation frequencies between 4 and 6% were measured in mammalian cells,[15, 16]whereas in *E. coli* cells only 1% mutation was found.[16, 17] The lower mutation frequencies in living cells compared to the primer extension experiments can be explained by the presence of repair systems.[18]

Our results indicate that polymerases Deep Vent exo$^-$ and Pwo generate G:C to T:A transversions at an 8-oxoguanine residue (Table 1). Several plasmids mutated at the *Sph*I site were sequenced. In agreement with an earlier report,[19] this result shows that one-mismatch ligations with four-nucleotide single-stranded overhangs can occur. Different findings were obtained at the *Bsm*I site, where we could not detect any mutations. Presumably, a mismatch in the two-nucleotide single-stranded overhang would prevent successful ligation and therefore only the correctly amplified sticky ends were selected. The data in Table 1 also show that the mutation frequencies with Deep Vent exo$^-$ and Pwo are very different from one another. An explanation for the high number of adenine incorporations by Deep Vent exo$^-$ might be its lack of a 3′,5′-exonuclease unit. The proofreading activity carried out by this unit is known to reduce the formation of mutations during DNA polymerization.[20, 21] The fact that polymerases Deep Vent exo$^-$ and Pwo incorporate both cytosine and adenine residues opposite 8-oxoguanine should not affect the applicability of the presented method because the mutations occur outside the coding region of the cloned gene.

Conclusion

Cloning efficiencies achieved by using chemical restriction at an 8-oxoguanine site are similar to those obtained by standard cloning with restriction enzymes and unmodified DNA strands. This chemical method offers an alternative to the standard method, by which only certain 3′-overhangs can be generated by restriction enzymes. A further advantage is that the sequence between the two restriction sites does not have to be considered in the choice of the strategy because cleavage will occur only at the modified nucleotide. The chemical method also guarantees cleavage very close to the end of a double-stranded DNA molecule. Therefore, primers containing 8-oxoguanine can be shorter than those used in the enzymatic method.

Experimental Section

Oligonucleotide synthesis: Oligodeoxyribonucleotides were synthesized in a DNA synthesizer (PerSeptive Biosystems, Expedite) by using standard phosphoramidite chemistry. For the 8-oxoguanosine modified oligonucleotides 8-oxy-5′-dimethoxytrityl-*N*2-isobutyryl-2′-deoxyguanosine-*O*-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite (Glen Research) was used. The final deprotection and cleavage step was performed over 8 h at 55 °C in aqueous ammonia (35%) containing 2-mercaptoethanol (0.25 M) to avoid oxidative degradation of the 8-oxoguanosine site.

DNA strand cleavage at 8-oxoguanosine: Oxygen was bubbled through the oligonucleotide solution for 5 min prior to treatment with ammonia, then the oligonucleotide 5′-CGCGXTACTGCT (**4**; 0.50 nmol; X = 8-oxoguanosine) was dissolved in aqueous ammonia (300 μL, 35% solution). The reactions were performed at various temperatures (60 – 90 °C) and with different reaction times (1 – 14 h). After evaporation of the liquid, the reaction mixture was resolved in nanopure water and analyzed by reversed phase HPLC. Identification of the products was performed by MALDI-TOF mass spectrometry.

PCR: A solution containing each primer (30 pmol), dATP, dCTP, dGTP, and TTP (20 nmol each; TP = triphosphate), pUC19 DNA (50 ng; New England Biolabs), and Deep Vent exo$^-$ (1.2 units; New England Biolabs) or Pwo polymerase (3.0 units; Roche) in reaction buffer (total volume 50 μL; Deep Vent exo$^-$: 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2.0 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8; Pwo: 10 mM Tris-HCl, 100 mM KCl, 60 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, pH 8.5) was incubated at 1) 95 °C for 45 s; 2) 55 °C for 55 s; 3) 72 °C for 2 min; steps (1) to (3) were repeated 25 times. The unmodified oligonucleotides 5′-TTGAATCATGCATGCCAGCTTGTCTGTAAGCGG and 5′-AATTTGATCGAATGCGTCAGTGAGCGAGGAAGCG, as well as the modified oligonucleotides 5′-CATXCGAGCTTGTCTGTAAGCGG (X = 8-oxoguanosine) and 5′-CXTCAGTGAGCG-AGGAAGCG were used for PCR.

Enzymatic restriction: A mixture of unmodified PCR product (50 μL), *Sph*I (10 units; New England Biolabs) and *Bsm*I (10 units; New England Biolabs) in reaction buffer (total volume 100 μL; 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1.0 mM dithiothreitol, pH 7.9) was incubated at 37 °C for 2 h. The sample was then kept at 65 °C for another 2 h. Purification was performed by preparative agarose gel electrophoresis.

Chemical restriction: Aqueous ammonia (300 μL, 35%) was added to the modified PCR product (20 μL) and oxygen was bubbled through the mixture for 5 min. Thereafter, the sample was kept at 60 °C for 14 h. Purification was carried out by Method A, B, or C.

Method A: The ammonia was evaporated and the volume of the mixture reduced to 100 μL. Annealing buffer (total volume 200 μL; 1.0 M NaCl, 100 mM KH_2PO_4, pH 6.0) was added. Annealing was performed by heating the sample to 95 °C and subsequent cooling to 25 °C in 1 h. Purification was performed by dialysis and membrane filtration.

Method B: The ammonia was evaporated and the volume of the mixture was reduced to 100 μL. Purification was performed by membrane filtration.

Method C: The ammonia was neutralized by cautious addition of 80% acetic acid (300 μL). Purification was performed by ethanol precipitation.

Preparation of the plasmid vector: A solution of pBR322 DNA (25 μg; New England Biolabs), *Sph*I (25 units), and *Bsm*I (25 units) in reaction buffer (total volume 200 μL; 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1.0 mM dithiothreitol, pH 7.9) was incubated at 37 °C for 14 h and at 65 °C for 3 h. Purification was performed by preparative agarose gel electrophoresis.

Ligation: Ligation was performed by incubation of insert DNA (10 μL), plasmid vector DNA (4 μL), and T4 DNA ligase (800 units; New England Biolabs) in reaction buffer (total volume 20 μL; 50 mM

Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1.0 mM dATP, 25 μg mL^{-1} bovine serum albumin, pH 7.5) at 16 °C for 16 h.

Transformation: An aliquot of *E. coli* DH5α cell suspension ($CaCl_2$ competent, 100 μL) was thawed on ice, the ligation solution (5 μL) was added and the sample was kept at 0 °C for 30 min. Heat shock was performed at 37 °C for 2 min. Thereafter, the sample was held at 0 °C for 30 min. After the addition of LB medium (1 mL), the suspension was shaken (200 rpF) at 37 °C for 1 h. The cells were then incubated on agar plates (25 μg mL^{-1} ampicillin, x-gal, IPGT) at 37 °C for 20 h. The cloning efficiency was determined by counting the blue cell colonies.

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