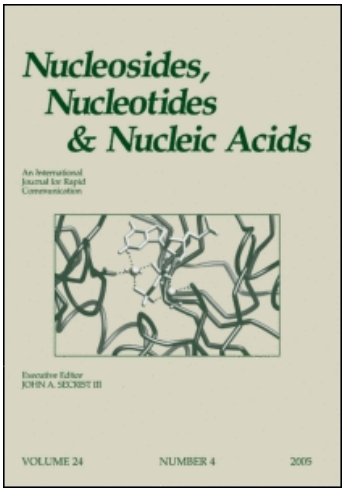


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# 8-HYDROXYDEOXYGUANOSINE IN DNA INHIBITS RESTRICTION ENDONUCLEASE DIGESTION

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**ABSTRACT** A DNA fragment containing 8-hydroxydeoxyguanosine was used to prime second strand synthesis of a recombinant pTZ19 phagemid single stranded DNA. The resultant double stranded closed circular product and also the annealed product of the single stranded DNA and the fragment were found to be resistant to cleavage by the restriction enzyme *Apa*LI in whose recognition sequence the 8-hydroxydeoxyguanosine had been inserted.



Oxygen radicals, which are probably agonists in the processes of mutagenesis, carcinogenesis and ageing (1) are formed in cells not only as the result of exogenous chemical treatment or radiation but also by intracellular metabolism. It has been shown that deoxyguanosine residues in DNA are hydroxylated at the C-8 position both *in vitro* and *in vivo* to produce 8-hydroxydeoxyguanosine (8-OH-dG) by various agents that produce oxygen radicals (2-6). It is possible, therefore, that the formation, by oxygen radicals, of 8-OH-dG in DNA is a cause of mutation, carcinogenesis and possibly ageing.

It is of interest to understand the effects 8-OH-dG incorporated into DNA has on both the chemical and biologial properties of DNA. It is known that 8-hydroxyguanosine favours the *syn* (7) rather than the more usual *anti* conformation. Although the total chemical structure of 8-OH-dG is not

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known, X-ray crystallographic studies on 9-ethyl-8-hydroxyguanine show that the purine skeleton of this molecule, in the crystal, is in the 6,8 diketo form (8). Also, studies on 8-hydroxyguanine using an ab initio molecular orbital method confirm the 6,8 diketo conformation, and show that the addition of an oxygen atom at position 8 changes entirely the electrostatic potential of the molecule giving it a negative character (9). It is likely, therefore, that in DNA the 8-OH-dG residue would be present mainly in the 8-keto form, with consequent changes to the biological properties of DNA. That this may be so is supported by the fact that when oligodeoxynucleotides containing an 8-OH-dG residue are used as templates for DNA polymerase I (Klenow fragment) catalysed DNA synthesis misreading takes place, not only at the 8-OH-dG residue itself, but also at adjacent pyrimidine residues (10).

In order to extend these studies and determine the effects 8-OH-dG residues in DNA may have on other enzymic activities associated with DNA, we report here the construction of a plasmid containing an 8-OH-dG residue in a restriction endonuclease recognition site and the resultant effect on restriction enzyme cleavage of DNA.



## MATERIALS AND METHODS

**Materials.** Chemicals used were of the highest purity available. DNA polymerase I (Klenow fragment) was from Amersham International plc, T4 DNA polymerase, T4 DNA ligase and T4 Gene 32 protein were from Bio-Rad Laboratories. *Acc*I, *Hpa*II, *Sph*I and *Hin*dIII were from Toyobo Co. Ltd. and *Apa*LI was from Takara Shuzo. The phagemid pTZ19R, the reverse primer and the helper phage M13K07 were from Pharmacia. *Escherichia. coli* TG1 cells were from Amersham.

**Methods.** *Preparation of 8-OH-dG-containing double-stranded insert.* The synthesis of the 8-OH-dG containing oligonucleotide (46-mer) has been described previously (10). A 19-mer (5'-GAGAAAGCTTGCATGCCTG) and a 49-mer (5'-GTAAAACGACCCGGGATGGTGCACTGATTGGCTGCAGGCATGCAAGCTT) were synthesized chemically using a DNA synthesizer (Applied Biosystems 380A). Equimolar amounts of the three oligonucleotides (200 pmole) in 110 µl 10 m*M* Tris-HCl pH 8.0, 5 m*M* $MgCl_2$ were annealed by heating at 60° for 1 hr and cooling slowly to room temperature, and then ligated at 4° overnight in the presence of 2 U T4 DNA ligase to form the double stranded insert (see Fig. 1).

*Preparation of recombinant pT2/46 DNA.* An aliquot of the ligation mixture containing approximately 31 pmole of the double stranded insert was

digested firstly with *Hpa*II (9 U) in low salt solution, followed by digestion with *Sph*I (10 U) in high salt solution, both digests being carried out at 37° for 1-2 hrs. Part of this digest (approx. 7.2 pmole) was ligated with *Acc*I and *Sph*I digested pTZ19R in the presence of T4 DNA ligase (1 U) in a total volume of 10 µl at 4° overnight, and the total ligation mixture was used to transform *E. coli* NM522 cells. Colonies containing the recombinant plasmid, pTZ/46, were selected by ampicillin resistance and inactivation of the lacZ' gene (white colonies). Plasmid DNA was prepared by standard procedures (11,12).

*Preparation of single stranded pTZ/46 DNA.* Single stranded (s.s.) circular DNA was prepared from colonies containing pTZ/46 phagemid following the procedure described by Mead *et al.* (13,14) using M13K07 as the helper phage. Using the reverse primer the sequence of the single stranded DNA in the insert and the surrounding region of the phagemid was determined.

A second preparation of this single stranded DNA was prepared by using the reverse primer for second strand synthesis catalysed by DNA polymerase I (Klenow fragment) and T4 DNA ligase. The double stranded (d.s.) DNA was then used to transform *E. coli* cells, strain TG1 (K12, Δ(*lac-pro*), *Sup*E, *thi*, *hsd* D5/F', *tra* D36, *pro*A+B+, *lac*q, *lac*ZΔM15). Colonies were selected from H-plates containing ampicillin (40 mg/l) and, from these, single stranded pTZ/46 DNA was isolated as described above. Again the sequence of the insert and the surrounding phagemid was determined.

*Annealing of 8-OH-dG containing oligonucleotide (A) and second strand synthesis.* After phosphorylation of the 5'-end in the presence of ATP and T4 polynucleotide kinase, oligonucleotide A (11.5 pmole) (see Fig. 1) was mixed with 1 pmole of single stranded pTZ/46 DNA in 10 µl solution containing 20 m*M* Tris-HCl pH 7.4, 2 m*M* $MgCl_2$ and 50 m*M* NaCl. The solution was heated for 1 hr at 70° and allowed to cool slowly to approx. 30°. This annealing reaction was carried out in duplicate. At this stage one reaction was stored at -20° and the second was kept on ice. To this latter sample 1.5 µl 10x synthesis buffer (100 m*M* Tris-HCl pH7.4, 50 m*M* $MgCl_2$, 20 m*M* dithiothreitol and 5 m*M* each of the four deoxynucleoside triphosphates), 2.5 U T4 DNA ligase, 1 U T4 DNA polymerase, and, in some reactions, 3 µg T4 gene 32 protein were added in a total volume of 15 µl. The solutions were kept on ice for 5 min., at 25° for 5 min. and incubated at 37° for 90 min. 85 µl TE buffer (10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA) was added and the solution was stored at -20°. The annealing and second strand synthesis reactions were also carried out using oligonucleotide B (the same as oligonucleotide A except that dG replaced 8-OH-dG) by exactly the same method.

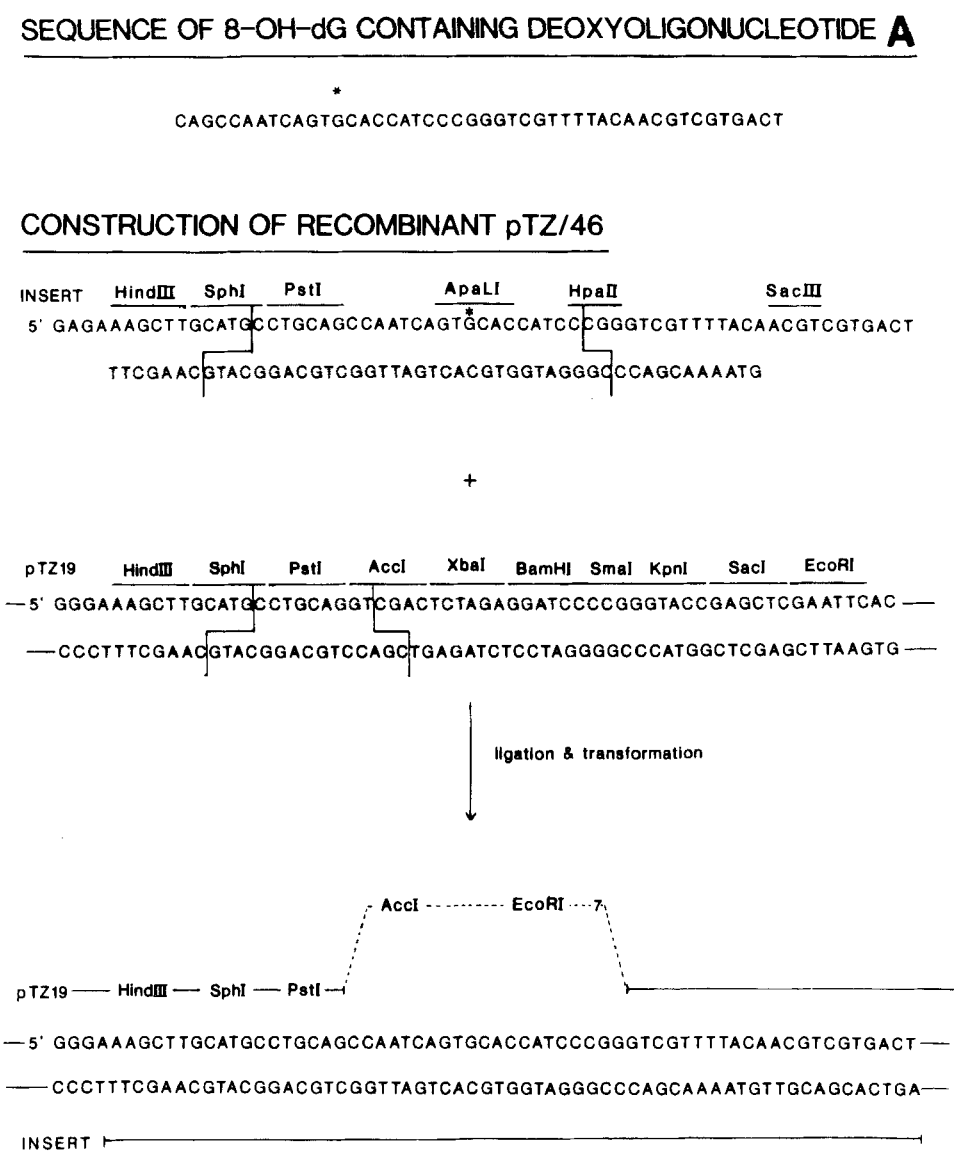



FIG. 1. Sequence of the 8-OH-dG containing 46-mer (oligonucleotide A, $\overset{*}{G}$=8-OH-dG) and the outline of the construction of the recombinant pTZ/46. The continuous lines above and below the bottom sequence signify regions of matching sequencing. (Oligonucleotide B has the same sequence as oligonucleotide A except that the 8-OH-dG residue is replaced by dG).

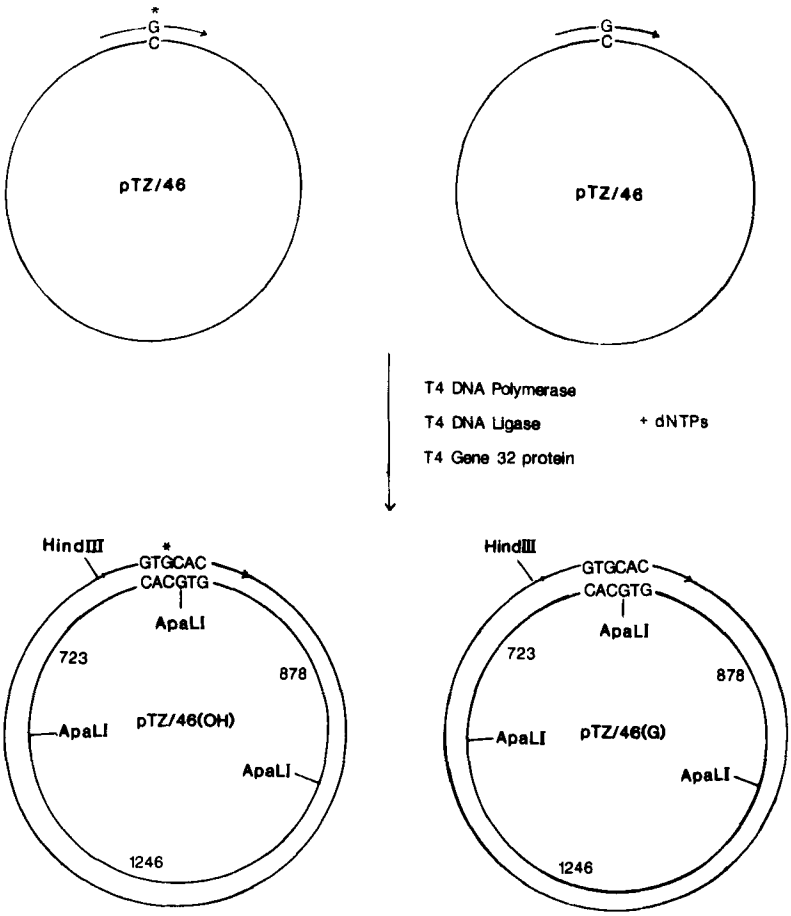
*Restriction enzyme digests.* One tenth of each of the reaction mixtures were incubated with either *Apa*LI (5 U) or *Hin*dIII (1 U) in 10 m*M* Tris-HCl pH 7.5, 10 m*M* $MgCl_2$, 50 m*M* NaCl and 1 m*M* dithiothreitol in a total volume of between 10 µl and 13 µl for 2 hrs at 37°. The reactions were stopped by adding 3 µl of a solution containing 20 m*M* Tris-HCl pH 8.0, 50 m*M* EDTA, 7 *M* urea, 0.05% bromophenol blue and 40% glycerol. The digests were run on 1% agarose gels at 50 V. *Apa*LI digests were run on gels in the absence of ethidium bromide and then stained in a solution containing ethidium bromide (5 µg/ml) for 5 min. *Hin*dIII digests and undigested samples of the annealing reactions and the synthesis/ligation reactions were run on gels containing ethidium bromide (0.5 µg/ml).

## RESULTS

**Construction of pTZ/46.** Fig. 1 shows the sequence of the 8-OH-dG containing 46-mer deoxyoligonucleotide (A) and the construction of the recombinant, pTZ/46. After ligation of the cleaved phagemid with the digested insert and transformation of NM522 cells, white colonies were selected and, from one of these, single stranded DNA was isolated. Using the reverse primer the sequence of this DNA was determined and the region at the site of recombination is shown at the foot of Fig. 1. The continuous lines above and below the sequence show where the sequences of pTZ19R and the insert DNA respectively match that of the isolated recombinant. It had been expected that the phagemid DNA isolated from white colonies would have the region between the *Sph*I and *Acc*I sites of pTZ19R replaced by the *Sph*I/*Hpa*II fragment of the synthetic insert DNA. It will be seen, however, that this is not the case, since downstream of the *Acc*I site the sequence is that of the insert rather than that of pTZ19R.

Although 8-OH-dG was present in the synthetic insert used in this construction, the sequencing showed that the recombinant isolated contained C in the complementary strand at this position. This complementary single-stranded DNA was used as the template for the preparation of 8-OH-dG containing d.s. DNA and also for control d.s. DNA where the standard G residue replaced the 8-OH-dG.

**Preparation of 8-OH-dG containing d.s. phagemid DNA *in vitro*.** The procedure used is illustrated in Fig. 2. The 5'-phosphorylated 8-OH-dG containing oligonucleotide A (see Fig. 1) was annealed to the complementary




EXPECTED SIZES OF ApaLI FRAGMENTS

| 8-OH-dG resistant | Wild-type |
|---|---|
| 1.6kb | |
| 1.2kb | 1.2kb |
| | 0.9kb |
| | 0.7kb |

FIG. 2. Products of annealing oligonucleotides A and B to pTZ/46 s.s. DNA and synthesis of cccDNAs pTZ/46/(OH) and pTZ/46(G) (G=8-OH-dG). Numbers inside the d.s. DNA indicate the number of b.ps. between the *Apa*LI sites.

strand of pTZ/46. Synthesis of the second strand was accomplished in the presence of the four deoxynucleoside triphosphates using T4 DNA polymerase, rather than DNA polymerase I (Klenow fragment), since it does not induce strand displacement (15) and, therefore, will not remove the 8-OH-dG containing oligonucleotide used as primer. The synthesis was carried out with and without the addition of T4 gene 34 protein, a single strand DNA binding protein, which assists T4 DNA polymerase synthesis in regions of secondary structure in a single-stranded template (16). As judged by gel electrophoresis of the products, the presence of the T4 gene 32 protein improved the second strand synthesis. The presence of T4 DNA ligase in the reaction gave a covalently closed circular DNA (cccDNA) as the major product [pTZ/46(OH)] (see Fig. 3).

In order to provide a 'control' cccDNA [pTZ/46(G)], similar reactions were carried out using, as the primer, oligonucleotide B in which dG replaces the 8-OH-dG residue.

**Restriction enzyme digestion of 8-OH-dG containing DNA.** The 8-OH-dG residue in pTZ/46(OH) is in one of the recognition sites of the restriction endonuclease *Apa*LI (GT$\overset{*}{G}$CAC; $\overset{*}{G}$=8-OH-dG). The *Hin*dIII site is 10 b.p. upstream of the 8-OH-dG residue. The *Hin*dIII site is a unique site whereas in pTZ/46 there are three *Apa*LI sites (including the one described above) as shown in Fig. 2.

The results of the digestions with *Apa*LI and *Hin*dIII are shown in Figs. 3 and 4, respectively. In Fig. 3 the right hand photograph shows the patterns obtained on agarose gel, in the absence of ethidium bromide, of the products after digestion with *Apa*LI. Three different samples of 8-OH-dG containing cccDNA and one sample of G containing cccDNA were digested with excess *Apa*LI. The G containing d.s. DNA[pTZ/46(G)] gave fragments of the expected sizes (as seen also for the digestion of pTZ/46 plasmid DNA) of 1.2 kb, 0.9 kb and 0.7 kb. The three samples of 8-OH-dG containing DNA[pTZ/46(OH)] all gave major fragments of 1.6 kb and 1.2 kb with two of the samples showing a very faint band corresponding to a fragment of 0.7 kb. One sample (that synthesised in the absence of T4 gene 32 protein) also shows a faint band at a position corresponding to 2.4 kb. The identity of this is unknown but it may represent a minor product derived from incomplete second strand synthesis. The products of annealing, after digestion with *Apa*LI gave single bands. However, the dG-containing product ran slightly more slowly than the 8-OH-dG-containing product, the latter's mobility being the same as for the

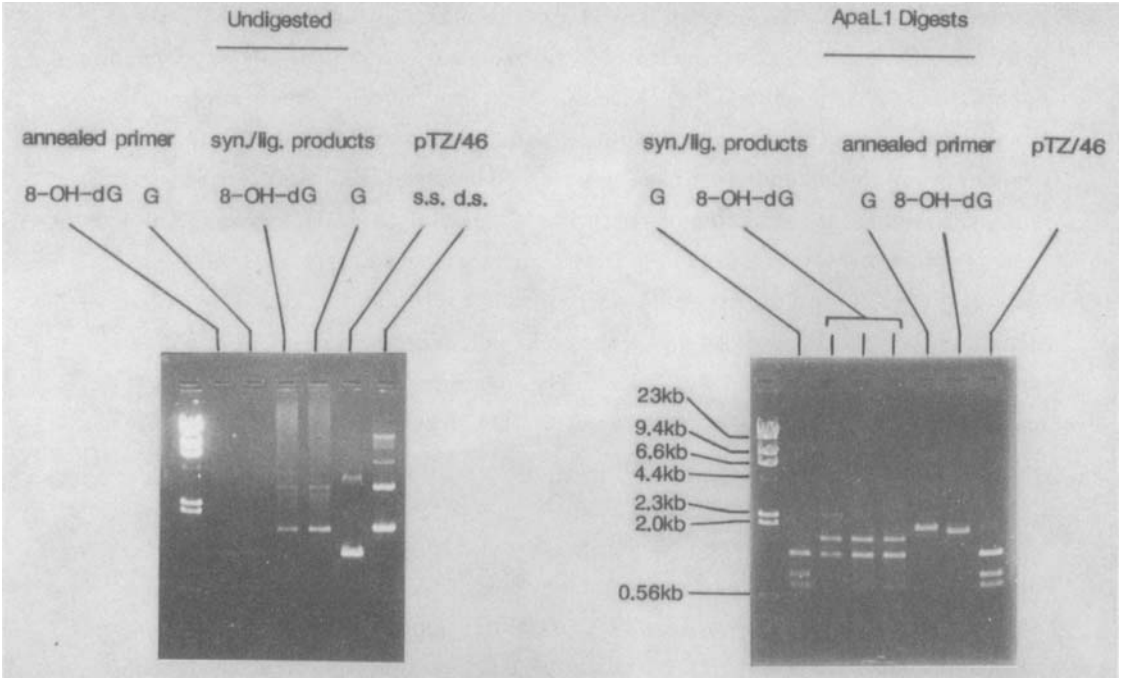



FIG. 3. Agarose gel electrophoresis (at 50 V) of undigested and *Apa*LI digested products of the annealing reactions and cccDNAs. The undigested products were run on an ethidium bromide containing gel, whereas the digested products were run on a gel in the absence of ethidium bromide. The extreme left hand lanes are size markers. In the right-hand photograph the syn./lig. products containing 8-OH-dG were prepared in the absence (lane 3 from the left) or presence (lanes 4 and 5, duplicate experiments) of T4 gene 32 protein.



undigested sample (not shown in the figure). In contrast to the digested samples, it will be seen in the left hand photograph that in the case of the undigested samples, for both the cccDNAs and the products of annealing (single faint bands) the patterns for the 8-OH-dG- and dG-containing samples are the same. It should be noted that this gel was run in the presence of ethidium bromide and hence the difference in mobilities of the annealed products compared to the right hand photograph.

These result indicate, therefore, that in both cccDNA and in DNA which is partially double stranded the presence of 8-OH-dG inhibits digestion by *Apa*LI.

Fig. 4 shows patterns obtained on agarose gel, run in the presence of ethidium bromide, for cccDNAs and the annealed products (single faint bands with identical mobilities) with and without digestion with *Hin*dIII. It will be

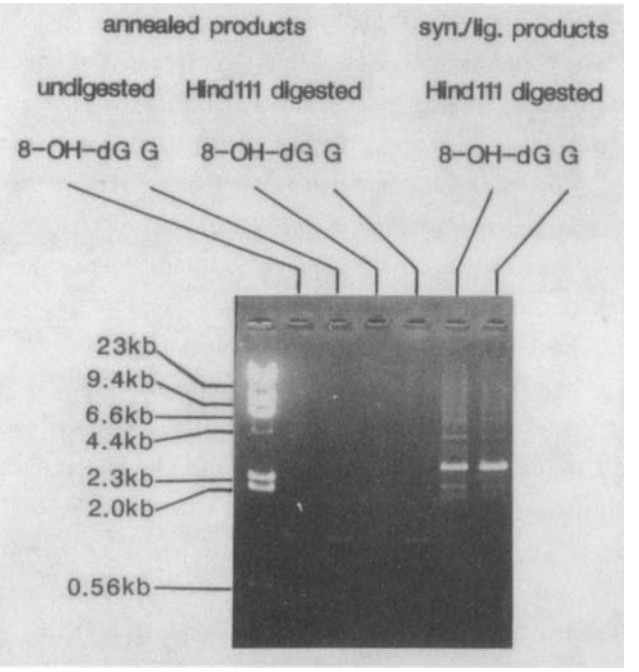



FIG. 4. Agarose gel electrophoresis (at 50 V) of undigested and *Hin*dIII digested products of the annealing reactions and cccDNAs. The gel was run in the presence of ethidium bromide. The extreme left hand lane contains size markers.



seen that: 1) products containing 8-OH-dG or dG run similarly; 2) the annealed products (with or without 8-OH-dG) are not cleaved by *Hin*dIII; and 3) that the major product after digestion of cccDNA is the predicted 2.8 kb linear DNA.

## DISCUSSION

The synthetic double stranded oligonucleotide containing 8-OH-dG and the vector, pTZ19, have matching sequences in the regions between 2 bp upstream of the *Hin*dIII site and the *Pst*I site, and between 8 bp and 29 bp downstream of the *Eco*RI site (see Fig. 1). Because of this it is difficult to locate precisely the points at which the insert has been ligated into the vector. As mentioned in the results the intention had been for the *Sph*I/*Hpa*II fragment of the synthetic double stranded oligonucleotide to

replace the *Sph*I/*Acc*I region of the vector, but sequencing has shown that this is not the case. Although it is possible that ligation of the insert and vector has taken place at the *Sph*I site, the *Sph*I/*Hpa*II fragment has not ligated into the vector's *Acc*I site. Rather, it appears that a recombination event has taken place between homologous regions of the insert and vector between 8 bp and 29 bp downstream of the vector's *Eco*RI site. It might be that recombination events have taken place in both homologous regions between the *Hin*dIII and *Pstl* sites, and downstream of the *Eco*RI site. The product obtained, however, contains the sequence required, i.e. a sequence complementary to the 8-OH-dG containing oligonucleotide A.

Digestion of the 8-OH-dG containing cccDNA [pTZ/46(OH)] were carried out with *Apa*LI and *Hin*dIII. This was done to distinguish effects caused by 8-OH-dG being present in the recognition site itself (*Apa*LI) and possible distal effects which might be detected by *Hin*dIII digestion. The *Hin*dIII digestion also demonstrated that second strand synthesis was complete since *Hin*dIII would not be expected to cleave single stranded regions of DNA. As well as carrying out digestions on the covalently closed circular pTZ/46(OH) or pTZ/46(G), digestions were carried out on the annealed products of single stranded pTZ/46 and oligonucleotides A or B. This was done for two reasons. Firstly to determine whether it might be local extra strain induced in the closed circular DNA rather than a direct effect of 8-OH-dG which might affect the cleavage efficiency. In the annealed product such strain would not be present. Secondly failure of digestion with *Hin*dIII confirms that single stranded DNA is not a substrate for the restriction enzyme.

By comparison of the results of digestion of pTZ/46(OH) and pTZ/46(G) with excess *Apa*LI it is clear that 8-OH-dG at the recognition site inhibits, almost totally, cleavage by the enzyme and the pattern of bands in the electrophoresis suggests that neither the 8-OH-dG-containing nor the complementary strand is cleaved. Since cleavage is also inhibited in the annealed product, it is likely that the 8-OH-dG is exerting a direct effect. This is also supported by the fact that in the closed circular DNA *Hin*dIII cleavage is not inhibited and there would, therefore, appear to be no distal affects caused by the introduction of conformational changes by 8-OH-dG.

Until 8-OH-dG is incorporated into other restriction enzyme recognition sequences we shall not know whether the inhibition seen here is unique to *Apa*LI or is more universal but, since it is known already that 8-OH-dG adopts a different conformation from the normal deoxynucleotides and it causes

misreading in DNA, it might be expected that its effects on restriction enzymes will be general. One consequence of this, at least in bacteria, is that the cell would be unable to use the restriction-modification system to remove 8-OH-dG containing DNA. Overall however, these results along with previously reported effects of 8-OH-dG suggest that the presence of 8-OH-dG in DNA has wide ranging effects on the properties and function of DNA.

## ACKNOWLEDGEMENTS

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