Nucleic Acids Research, 1994, Vol. 22, No. 18 3737–3741

Inefficient excision of uracil from loop regions of DNA oligomers by *E.coli* uracil DNA glycosylase

N.Vinay Kumar and U.Varshney*
Centre for Genetic Engineering, Indian Institute of Science, Bangalore 560012, India

Received May 31, 1994; Accepted July 26, 1994

ABSTRACT

Kinetic parameters for uracil DNA glycosylase (*E.coli*)-catalysed excision of uracil from DNA oligomers containing dUMP in different structural contexts were determined. Our results show that single-stranded oligonucleotides (unstructured) are used as somewhat better substrates than the double-stranded oligonucleotides. This is mainly because of the favourable V_{max} value of the enzyme for single-stranded substrates. More interestingly, however, we found that uracil release from loop regions of DNA hairpins is extremely inefficient. The poor efficiency with which uracil is excised from loop regions is a result of both increased K_m and lowered V_{max} values. This observation may have significant implications in uracil DNA glycosylase-directed repair of DNA segments that can be extruded as hairpins. In addition, these studies are useful in designing oligonucleotides for various applications in DNA research where the use of uracil DNA glycosylase is sought.

INTRODUCTION

Uracil DNA glycosylases (UNG) excise uracil residues from DNA (1–3). Uracil residues in DNA arise as a result of deamination of cytosines or incorporation of dUMP by DNA polymerase. However, the latter is kept to a minimum by the presence of dUTPase (*dut*) which maintains low levels of dUTP in the cell (1–3). UNG uses single-stranded substrates more efficiently than double-stranded substrates (4–7). However, it was also reported (8) that uracil residues from some regions of single-stranded M13 DNA were removed inefficiently. This observation suggests that certain regions of genome may be prone to G:C to A:T transition mutations because of inefficient UNG-directed DNA repair.

We are using dUMP-containing synthetic oligonucleotides as model substrates to understand the mechanism of enzyme action. Earlier we used single-stranded DNA oligomers of different sizes containing dUMP in varying positions and showed that *E.coli* UNG excises even those uracil residues located near the ends of the single-stranded DNA (9). Such studies are important not only in considering the details of the mechanism of enzyme action but also in increasing the usefulness of UNG in DNA research (10–16).

In this study, we have determined kinetic parameters of uracil release from DNA oligomers containing dUMP in three different structural contexts: single-stranded, double-stranded and stem and loop regions of hairpins. We show that excision of uracil from loop regions of hairpin structures is very poor. Furthermore, our findings also provide a rationale as to why uracil residues in some regions of the genome may be removed with a poor efficiency.

MATERIALS AND METHODS

Oligodeoxyribonucleotides (oligonucleotides)

These were obtained from the Regional DNA Synthesis Laboratory at the University of Calgary (Calgary, Canada) and the DNA synthesis facility at the Centre for Genetic Engineering, Indian Institute of Science (Bangalore, India). A list of oligonucleotides, their sizes and various abbreviations used to denote them is given in Table 1. U-loop and U-stem oligonucleotides which fold into hairpin structures were purified by HPLC on a PRP-1 column under strong alkaline (pH 12.7) conditions (17), while others were gel purified (15% polyacrylamide–8 M urea) and passed through Sephadex G-50 columns to remove salts.

Radioisotopes, enzymes and chemicals

Radioisotopes were from BARC (India) and enzymes were from Boehringer Mannheim (BM) (Germany) or Bangalore Genei (India). Uracil DNA glycosylase was purified from *E.coli* (4,18). Chemicals (AR grade) were from Sigma (USA) or SRL (India).

^{32}P-labelling, purification and quantitation of oligonucleotides

To study the kinetics of uracil excision, quantitative 5′ ^{32}P-end-labelling was performed as described (9) except that low specific activity [γ-^{32}P]ATP was prepared by diluting 8.0 μl of 3500 Ci/mmole (2.85 μM) [γ-^{32}P]ATP with 72 μl of 100 μM cold ATP. U-loop and U-stem oligonucleotides were also labelled by filling in of the 3′ recessed ends by Klenow polymerase in the presence of excess low specific activity [α-^{32}P]dCTP prepared by diluting 20 μl of [α-^{32}P]dCTP 3000 Ci/mmole (3.33 μM)

*To whom correspondence should be addressed.

with 60 μl of cold dCTP (100 μM) (19) and quantitated. This was done to allow a better separation of the substrate and the product. The ^{32}P-labelled oligonucleotides were purified by chromatography on Sephadex G-50 minicolumns.

Formation of double-stranded oligonucleotides

5′ ^{32}P-End-labelled fp-KRS and rp-ung oligonucleotides were mixed with 2.5 molar excess of complementary oligonucleotides in distilled water. The tubes were heated at 90°C for 5 min, supplemented with 10× UNG buffer to adjust the final concentration to 1× (see below) and left at 4°C for 4 h. Aliquots were analysed on a 12% polyacrylamide gel under non-denaturing condition in TBM buffer (90 mM Tris−HCl, 90 mM boric acid, 5 mM $MgCl_2$) (20) prior to use.

Uracil DNA glycosylase (UNG) reactions

Standard conditions. Oligonucleotides (10 pmol) were 5′ ^{32}P-end-labelled with 5 μCi of high specific activity (3500 Ci/mmole) [γ-^{32}P]ATP and T4 polynucleotide kinase in 10 μl reactions. Aliquots (1 μl) were used for UNG reactions. Reaction mixture (15 μl) consisting of 50 mM Tris−HCl (pH 7.4), 1 mM Na_2EDTA, 1 mM DTT and 25 μg/ml BSA (BM) was supplemented with 10−20 ng of UNG and incubated at 37°C for 1 h. Reactions were stopped by adding 15 μl of 0.1 M NaOH and chilling on ice. Cleavage at abasic sites was effected by heating at 90°C for 30 min. Contents were dried in a Speedvac (Savant) and dissolved in 10 μl loading buffer (80% formamide, 0.1% xylene cyanolFF and bromophenol blue, and 1 mM Na_2EDTA). Aliquots (5 μl) were analysed on 15% polyacrylamide−8 M urea gels (21) of 0.8 mm thickness and exposed to Indu X-ray films (Hindustan Photo Films, India) with or without hyperscreens (Amersham) for 1−2 h and visualized by autoradiography. Control reactions were treated in a similar manner except that no enzyme was added.

Determination of K_m and V_{max} values. Reactions (15 μl) containing varying amounts of quantitatively ^{32}P-labelled substrates (0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 15 pmol) were carried out as described with different concentrations of enzyme (determined from range finding experiments). Reactions were incubated at 37°C for 10 min, mixed with an equal volume of 0.1 M NaOH and processed as above. Gel pieces corresponding to the product and the remaining substrate were cut out and counted in a scintillation counter (LKB). For accurate measurements, fractions calculated as $A/(A+B)$, where A is the number of counts in the band corresponding to the product and B for the remaining substrate, were used to determine the amount of product. Values of K_m and V_{max} were determined from Hofstee plots (22) of two independent experiments.

RESULTS

Oligonucleotides and their characterization

A list of oligonucleotides and various abbreviations used to denote them is given in Table 1. Figure 1 shows an autoradiograph of 5′ ^{32}P-end-labelled oligonucleotides electrophoresed on 15% polyacrylamide−8 M urea gel. Most of the oligonucleotides migrate as single bands indicating their purity; diffused bands seen in the case of U-loop and U-stem (Figure 1, lanes 6 and 7) are due to incomplete denaturation of these oligonucleotides (Materials and Methods). However, under non-denaturing conditions, these oligonucleotides migrate as single bands (Figure 2, lanes 1 and 2). Figure 2 also shows the results of annealing of 5′ ^{32}P-end-labelled fp-KRS with *anti*-fp-KRS (duplex 1) (lanes 4 and 5) and 5′ ^{32}P-end labelled rp-ung with *anti*-rp-ungG (duplex 2G) (lanes 7−9) or *anti*-rp-ungA (duplex 2A) (lanes 10−12). Results show that when complementary oligonucleotides were present in equimolar to 2.5-fold molar excess, all of the 5′ ^{32}P-end-labelled oligonucleotide was driven into duplexes (compare lanes 4 and 5 with 3; lanes 7−12 with 6). Therefore, a 2.5-fold molar excess of the complementary oligonucleotide was routinely used to prepare double-stranded substrates for UNG reactions. Line diagrams representing secondary structure of the oligonucleotides are shown in Figure 3.

Table 1. List of the oligonucleotides

S. No.	Oligonucleotide	Size (nt)	Sequence
1	fp-KRS	27	d(agcUcgccgacgaatatgatacaggag)
2	*anti*-fp-KRS	27	d(cacctgtatcatattcgtcggcgagct)
3	U-loop	32	d(cgatctagaggatcctUttggatcctctagat)
4	U-stem	32	d(cgatctagaggatcctttttggatccUctagat)
5	rp-ung	19	d(tgccaUccggcatttcccc)
6	*anti*-rp-ungG	19	d(ggggaaatgccgggtggca)
7	*anti*-rp-ungA	19	d(ggggaaatgccggatggca)

Abbreviations: fp-, forward primer; rp-, reverse primer; KRS-, lysyl-tRNA synthetase; *anti*-, complementary to; U-loop and U-stem denote presence of dUMP in loop or stem regions of these hairpins. Underscored sequence within these oligonucleotides form stems of the hairpin structures. *Anti*-rp-ungG and *anti*-rp-ungA denote the oligonucleotides complimentary to rp-ung; duplexes formed upon annealing them with rp-ung contain U paired with G or A, respectively. Location of dUMP in the oligonucleotides is shown by U in capital letters.

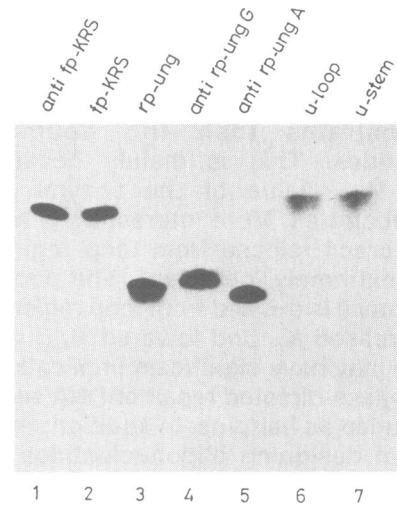

Figure 1. Electrophoresis of 5′ ^{32}P-end-labelled oligonucleotides on a denaturing 15% polyacrylamide−8 M urea gel. Names of the oligonucleotides are shown on top of the lanes.

UNG reactions

UNG reaction on dUMP-containing oligonucleotides generates abasic sites which are sensitive to cleavage under alkaline conditions. As a result of this cleavage two fragments are obtained, one of which (^{32}P-labelled) is detected as a product. As is evident from Figure 4, a product of expected size is seen in all reactions. Products in lanes 2 and 4 appear to migrate faster

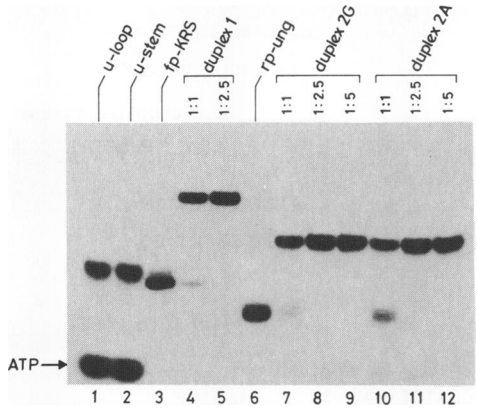

Figure 2. Electrophoresis of various substrates on a non-denaturing 12% polyacrylamide gel. Names of oligonucleotides are shown on top of each lane. Molar ratios of the respective complementary oligonucleotides to form duplex 1 from fp-KRS (lane 3–5) and duplex 2G and duplex 2A from rp-ung (lanes 6–12) are also shown.

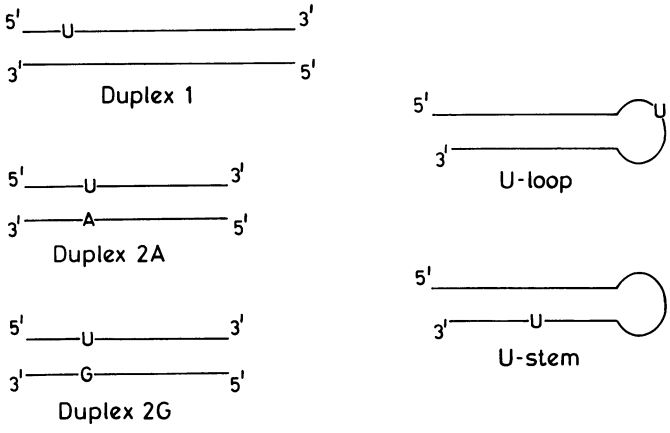

Figure 3. Diagrammatic representation of secondary structures of the oligonucleotides used in this study.

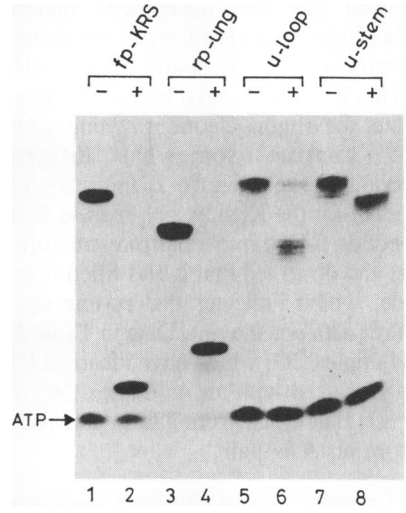

Figure 4. Analysis of UNG reaction products on a 15% polyacrylamide–8 M urea gel. Reactions were performed with (+) or without (–) UNG. Names of the oligonucleotides are shown on top of the lanes.

Table 2. Kinetic parameters of uracil excision from various substrates

S. No.	Substrate	K_m (×10^{-7} M)[a]	V_{max}[b]	V_{max}/K_m	Relative V_{max}/K_m[c]
1	fp-KRS	5.0	26.9	5.4	100
2	Duplex 1	2.4	4.3	1.8	33
3	fp-KRS(NC)	3.3	20.1	6.1	112
4	rp-Ung	15.8	17.3	1.1	20
5	Duplex 2A	1.8	7.2	4	74
6	Duplex 2G	1.9	9.6	5	96
7	U-loop	9.5	1.2	0.12	2
8	U-stem	2.2	7.0	3.2	59

All values are average of 2 independent experiments. Duplex 1 was formed by annealing fp-KRS with 2.5 molar excess of *anti*-fp-KRS; fp-KRS(NC) denotes fp-KRS mixed with 2.5 molar excess of *anti*-rp-ungG; duplex 2A and duplex 2G were formed by annealing 2.5 molar excess of rp-ung with *anti*-rp-ungA and *anti*-rp-ungG, respectively

[a]K_m values are for the uracil residue in the oligonucleotides.

[b]V_{max} values are in picomol/min/μg protein.

[c]Relative V_{max}/K_m are shown in % with respect to fp-KRS. Values have been shown to the nearest complete number.

than their sizes of a trimer or a pentamer. This was expected because hydrolysis of the phosphodiester bond at the abasic sites results in 3′ terminal phosphates to the products conferring significantly faster mobility to these small oligonucleotides (9). The products of U-loop and U-stem (lanes 6 and 8, respectively) migrate as diffused bands. This is most likely because the product of U-stem could still retain the hairpin structure (5′ cgatctagaggatccttttggatcc 3′) and the 15mer 5′ ^{32}P-end-labelled product released from U-loop can again fold into an alternate hairpin structure (5′ cgatctagaggatcct 3′).

Excision of uracil from fp-KRS and duplex 1

UNG reactions on single-stranded and double-stranded oligonucleotides were performed to determine the K_m and V_{max} values (Table 2). Relative V_{max}/K_m values for fp-KRS and duplex 1 show that excision of uracil from a single-stranded context is about 3-fold better than its excision from a double-stranded context. To see if the reduction in efficiency of uracil release from duplex 1 was a result of increased DNA concentration (because 2.5 molar excess of the complementary oligonucleotide was used to drive fp-KRS into duplex 1), we performed kinetics on fp-KRS in the presence of 2.5 molar excess of *anti*-rp-ungG (not complementary to fp-KRS). Although the K_m and V_{max} values were slighty changed, the V_{max}/K_m value did not change significantly [Table 2, fp-KRS(NC)]. Thus the lowered release of uracil from duplex 1 is mainly because of its presence within the double-stranded context.

Excision of uracil from rp-ung, duplex 2A and duplex 2G

Contrary to fp-KRS, release of uracil from rp-ung is slow but increases when it is driven into duplexes with either *anti*-rp-ungA (duplex 2A) or *anti*-rp-ungG (duplex 2G) (Table 2). This observation suggests that poor release of uracil from rp-ung is unlikely to be a result of sequence context *per se* (in which the dUMP is located) but is a result of some secondary structure into which the dUMP residue is sequestered. Analysis of the rp-ung

sequence showed that the nucleotides underlined in this oligonucleotide [d(tgccaUccggcatttcccc)] are complementary and capable of forming a hairpin structure with the dUMP in a tetra-loop region. This is also indicated by the fact that despite being of the same size, the oligonucleotides rp-ung, *anti*-rp-ungG and *anti*-rp-ungA migrate somewhat differently on a urea−polyacrylamide gel (Figure 1, lanes 3−5). Further, we used *anti*-rp-ungG for the Klenow polymerase filling reaction (if this oligonucleotide folded into a hairpin structure it would have a 5′ overhang) and observed that it was filled in to give a 26mer oligonucleotide, which indicates that rp-ung could fold into a hairpin structure (data not shown). Data in Table 2 obtained with duplex 2A and duplex 2G, which have identical DNA sequences except having U paired with an A (duplex 2A) or a G (duplex 2G) also suggests that uracil from a G:U pair is excised slightly better than from an A:U pair.

Excision of U residues from the loop region of hairpins

To confirm the observations with rp-ung, we carried out kinetic studies with U-loop and U-stem oligonucleotides which fold into tetra-loop hairpin structures. Both of these oligonucleotides have the same DNA sequence except that the U-loop contains a U in the loop region and the U-stem in the stem region. As shown in Table 2, the excision of U from U-stem oligonucleotide is efficient (59%) but its release from U-loop is substantially decreased (~2%). The extremely poor efficiency of uracil release from tetra-loop regions of a hairpin is a result of both increased K_m and decreased V_{max} values (Table 2).

To obtain additional evidence that poor excision of uracil from loop regions is a consequence of structural context and not of the neighbouring sequence *per se*, we utilized U-stem for UNG reactions as shown in Figure 5. The 3′ recessed end of U-stem was filled in by Klenow polymerase in the presence of [α-^{32}P]dCTP and subjected to digestion with *Bam*HI. This leads to release of a dUMP-containing 14mer oligonucleotide (U-loopUS) which can fold into yet another stem−loop structure containing dUMP in a penta-loop region.

A time−course kinetics of uracil excision from U-stem containing the dUMP residue in the stem region, and from the U-loopUS containing the same dUMP residue in the loop region is shown in Figure 6. It is evident from this figure that excision of uracil from U-stem is efficient (lanes 2−6), but excision of the same uracil residue is substantially lowered when brought into the structural context of a loop in U-loopUS (compare lanes 2−6 with lanes 8−12).

DISCUSSION

Comparision of K_m and V_{max} values for fp-KRS and duplex 1 (Table 2) suggests that single-stranded DNAs (unstructured) are somewhat better substrates than the double-stranded DNA mainly as a consequence of higher V_{max} values for the enzyme. Using single-stranded DNA oligomers, we earlier showed that the efficiency of uracil excision slightly increased as its location was moved inwards from the 5′- terminal position (9). Hence, difference in the efficiencies (V_{max}/K_m) with which uracil is excised from double-stranded regions (duplex 1 and U-stem, Table 2) could be due to the location of dUMP within these oligonucleotides. It was recently shown that within the double-stranded substrates, release of uracil from a G:U wobble base pair was slightly better than that from an A:U base pair (23). Our results with a different set of double-stranded oligonucleotides

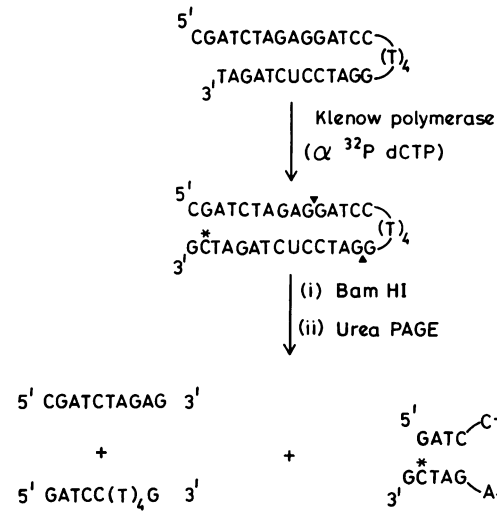

Figure 5. Outline for preparation of 14mer oligonucleotide (U-loopUS) from U-stem. A solid triangle shows *Bam*HI cutting sites and the asterisk indicates position of ^{32}P-radiolabelled nucleotide.

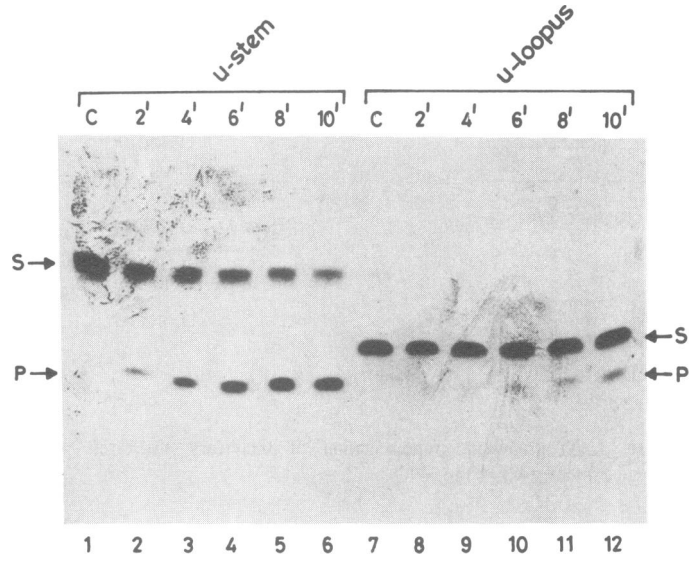

Figure 6. Kinetics of uracil excision from U-stem and U-loopUS. UNG reactions on each substrate (10,000 c.p.m.) were carried out for times indicated with 30 pg/ml enzyme dilution. Products were analysed on a 12% polyacrylamide−8 M urea gel and autoradiogaphed. The substrate (S) and the product (P) bands are indicated with arrows.

are in complete agreement with this finding. Slightly better release of uracil from a G:U pair is also relevent from physiological considerations where cytosine deamination will result in a G:U pair. Thus a more recent report (7) showing somewhat less efficient excision of uracil from a G:U pair (compared to an A:U pair) is still not understood.

During these studies we found that excision of uracil from tetra-loop regions of hairpin structures (U-loop, rp-ung, Table 2) and a penta-loop region (U-loopUS, Figure 6) is very inefficient. This observation could be of significant importance in UNG-directed repair of the DNA regions that can be extruded as hairpins. Comparision of the kinetic parameters (Table 2) shows that rp-

ung is a better substrate than U-loop. Exact details of the base interactions within the loop region of hairpins are influenced by the sequence in the loop as well as by the sequence of the stem region (25,26). Thus, besides the difference in the stability of the hairpins, conformational differences in the sugar–phosphate backbone and the interactions within the loop bases may also explain why rp-ung is a better substrate than U-loop.

DNAs containing UMP (instead of dUMP) are not substrates for UNG (24, our unpublished results) indicating that both sugar and the base moieties are recognized by the enzyme. Thus a first step in the recognition of dUMP by the enzyme could be the local melting of the DNA. For single-stranded DNA lacking secondary structures, melting will not be required and can be easily achieved for a double-stranded form compared to the loop regions of the hairpins (27) or other sharp fold-back structures (28). As the single-stranded nucleic acids can be associated with unique and not readily predictable secondary or complex tertiary structures, the present findings provide a rationale to why some of the dUMP residues are poorly cleaved from single-stranded M13 DNA (8). In fact, short stretches of complementary sequences do flank many of these dUMP residues. Taken together, our results clearly suggest a definite role of DNA structure on uracil excision by UNG.

In addition, our findings should also be of general use for designing of oligonucleotides, etc. for various molecular manipulations in DNA research where use of UNG is sought. Recently, based on the finding that UNG efficiently excises uracil from the ends of double-stranded DNA substrates also, we developed an efficient method for cloning of PCR-amplified DNA fragments (29).

ACKNOWLEDGEMENTS

We are thankful to Prof. J.H.van de Sande (University of Calgary, Calgary, Canada) for his support in carrying out this work. We thank the oligonucleotide synthesis facilities at the University of Calgary and at CGE, IISc, Bangalore, India for synthesis of oligonucleotides and Dr D.N.Rao and our other colleagues for their critical comments on the manuscript. This research was funded by grants from the DBT and DST of India to U.V. and a CSIR studentship to N.V.K.

REFERENCES

1. Duncan,B.K. (1981) In *The Enzymes* (Boyer, P., ed), pp 565–586, Academic Press, Orlando, FL.
2. Lindahl,T. (1982) *Annu. Rev. Biochem.* **51**, 61–87.
3. Sakumi,K. and Sekiguchi,M. (1990) *Mutat. Res.* **236**, 161–172.
4. Lindahl,T., Ljungquist,S., Siegert,W., Nybert,B., and Spevens,B. (1977) *J. Biol. Chem.* **252**, 3286–3294.
5. Krokan,H. and Wittwer,C.U. (1981) *Nucleic Acids Res.* **9**, 2599–2613.
6. Leblanc, J.-P and Laval, J. (1982) *Biochimie* **64**, 735–738.
7. Eftedal,I., Guddal,P.H., Slupphaug,G., Volden,G. and Krokan,E. (1993) *Nucleic Acids Res.* **21**, 2095–2101.
8. Delort,A.-M, Duplaa,A.-M, Molko,D. Teoule,R., Leblanc,J.-P. and Laval,J. (1985) *Nucleic Acids Res.* **13**, 319–335.
9. Varshney,U. and van de Sande,J.H. (1991) *Biochemistry* **30**, 4055–4061.
10. Craig,G., Nizetic,D. and Lehrach,H. (1989) *Nucleic Acids Res.* **17**, 4605–4610.
11. Longo,M.C., Berninger,M.S. and Hartley,J.L. (1990) *Gene* **93**, 125–128
12. Ball,J.K. and Desselberger,U. (1992) *Nucleic Acids Res.* **20**, 3255.
13. Pu,W.T. and Struhl,K. (1992) *Nucleic Acids Res.* **20**, 771–775.
14. Rashtchian,A., Buchman,G.W., Schuster,D.M. and Berninger,M.S. (1992) *Anal. Biochem.* **206**, 91–97.
15. Devchand,P.R., McGhee,J.D. and van de Sande,J.H. (1993) *Nucleic Acids Res.* **21**, 3437–3443.
16. Smith,C., Day,P.J.R. and Walker,M.R. (1993) *PCR Methods Appl.* **2**, 328–332.
17. Germann,M.W., Pon,R.T. and van de Sande,J.H. (1987) *Anal. Biochem.* **165**, 399–405.
18. Varshney,U., Hutcheon,T., and van de Sande,J.H. (1988) *J. Biol. Chem.* **263**, 7776–7784.
19. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition. Cold Spring Harbor, NY.
20. Van de Sande,J.H., Ramsing,N.B., Germann,M.W., Elhorst,W., Kalisch,B., Kitzing,E.v., Pon,R.T., Clegg,R.C. and Jovin,T.M. (1988) *Science* **241**, 551–557.
21. Maxam,A.M. and Gilbert,W.A. (1980) *Methods Enzymol.* **65**, 499–560.
22. Dowd,J.E. and Riggs,D.G. (1965) *J. Biol. Chem.* **240**, 863–869.
23. Verri,A., Mazzarello,P., Spadari,S. and Focher,F. (1992) *Biochem. J.* **287**, 1007–1010.
24. Talpaert-Borle,M. Campagnari,F. and Creissen,D.M. (1982) *J. Biol. Chem.* **257**, 1208–1214.
25. Zhou,N. and Vogel,H.J. (1993) *Biochemistry* **32**, 637–645.
26. Blommers,M.J.J., van der Marcel,G.A., von Boom,J.H., and Hilbers,C.W. (1989) *Biochemistry* **28**, 7491–7498.
27. Antao,V.P., Lai,S.Y. and Tinoco,Jr I. (1992) *Nucleic Acids Res.* **19**, 5901–5905.
28. Hirao,I., Kawai,G., Yoshizawa,S., Nishimura,Y., Ishido,Y., Watanabe,K. and Miura,K. (1994) *Nucleic Acids Res.* **22**, 576–582.
29. Vinay Kumar,N. and Varshney,U. (1994) submitted for publication.