Excision of uracil from the ends of double stranded DNA by uracil DNA glycosylase and its use in high efficiency cloning of PCR products

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We show that uracil DNA glycosylase from *E. coli* excises uracil residues from the ends of double stranded oligos. This information has allowed us to develop an efficient method of cloning PCR amplified DNA. In this report, we describe use of this method in cloning of *E. coli* genes for lysyl- and methionyl-tRNA synthetases. Efficiency of cloning by this method was found to be the same as that of subcloning of DNA restriction fragments from one vector to the other vector. Possibilities of using other DNA glycosylases for such applications are discussed.

Uracil DNA glycosylases (UDGs) excise uracil residues from DNA. Using dUMP containing viral DNAs, it was shown that UDGs use single stranded substrates more efficiently than the double stranded substrates. Earlier we used single stranded DNA oligomers of different sizes containing dUMP in varying positions and showed that *E. coli* UDG excises even those uracil residues located near the ends of the single stranded DNA. Recently, UDG has become an important enzyme in DNA research. To further extend the applications of UDG, we determined efficiency of uracil release from the ends of double stranded oligos. Based on the finding that UDG removes uracil residues located near the ends of double stranded DNA, we designed an efficient method for cloning of PCR amplified DNA. Here we describe use of this method in cloning *E. coli* genes for lysyl- and methionyl-tRNA synthetases.

Materials and methods

Oligodeoxyribonucleotides (oligos)

These were obtained from the Regional DNA Synthesis Laboratory at the University of Calgary, Calgary, Canada or from the Oligo Synthesis Facility at CGE, IISc, Bangalore. A list of oligos used in this study, their sizes and various abbreviations used to denote them are given in Table 1. All of the oligos 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, Germany (BM) or Bangalore Genei, India. Uracil DNA glycosylase was purified from *E. coli*. Chemicals (AR grade) were from Sigma, USA or SRL, India.

[^32]P-labelling, purification and quantitation of oligos

To study the kinetics of uracil excision, quantitative [^32]P-end-labelling was performed as described in ref. 8, except that low specific activity [~[^32]P~] ATP was prepared by diluting 8.0 ml of 3500 Ci/mmmole (2.85 mM) [~[^32]P~] ATP with 72 ml of 100 mM cold ATP. The [^32]P-labelled oligos were purified by chromatography on Sephadex G-50 minicolumns.

Formation of double stranded oligos

[^32]P-end-labelled fp-KRS was mixed with equimolar or 2.5 molar excess of complementary oligos in distilled water. The tubes were heated at 90°C for 5 min, supplemented with 10 x UDG-buffer to adjust the final

<table>
<thead>
<tr>
<th>Table 1. List of the oligos</th>
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<tbody>
<tr>
<td><strong>Oligo</strong></td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>fp-MRS</td>
</tr>
<tr>
<td>rp-MRS</td>
</tr>
<tr>
<td>fp-KRS</td>
</tr>
<tr>
<td>rp-KRS</td>
</tr>
<tr>
<td>anti rp-KRS</td>
</tr>
<tr>
<td>anti MRS</td>
</tr>
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</table>

Abbreviations: fp-, forward primer; rp-, reverse primer; MRS-, methionyl-tRNA synthetase; KRS-, lysyl-tRNA synthetase, anti-, complementary to; Anti rp-ungG denotes the oligos complementary to rp-ung (not shown). Location of dUMP in the oligos is shown by U in bold letters.

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concentration to 1 x (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 MgCl₂) (ref. 16) prior to use.

Uracil DNA glycosylase (UDG) reactions

Standard conditions. Oligos (10 pmole) were 5'-end-labelled with 5 μCi of high specific activity (3500 Ci/m mole) [γ-32P] ATP and T4 polynucleotide kinase in 10 μl reactions. Aliquots (1 μl) were used for UDG reactions. Reaction mixture (15 μl) consisting of 50 mM Tris.HCl (pH 7.4), 1 mM Na₂EDTA, 1 mM DTT and 25 μg/ml BSA (B.M.) was supplemented with 10–20 ng of UDG 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 Speed vac (Savant) and dissolved in 10 μl loading buffer (80% formamide, 0.1% xylene cyanol FF and bromophenol blue, and 1 mM Na₂EDTA). Aliquots (5 μl) were analysed on 15% polyacrylamide-8 M urea gels17 of 0.8 mm thickness and exposed to Indu X-ray films (Hindustan Photo Films, India) with or without hypo screens (Amersham) for 1 to 2 h and visualized by autoradiography. Control reactions were treated in a similar manner except that no enzyme was added.

Uracil DNA glycosylase reactions for kinetics of uracil release. Oligomer, fp-KRS was quantitatively 5'-end-labelled and purified on G-50 columns. Reactions (70 μl) containing 7 pmole of substrate (in single stranded or double stranded form) were carried out as described above except that an appropriate dilution of enzyme (determined from a range finding experiment) was added and aliquots (10 μl) were removed at various time intervals (0, 2, 4, 6, 8, 10 min), mixed with equal volume of 0.1 M NaOH and processed as above. Regions of gel corresponding to the unreacted substrate and the product were cut out and counted in a scintillation counter (LKB). Percent cleavage efficiencies were calculated as [100 X (B/A + B)], where A is the number of counts in the band representing unreacted substrate and B is the number of counts in the band corresponding to product, and plotted on Y axis versus time on X axis. Slopes of the straight lines were determined and percentage efficiencies of uracil release relative to fp-KRS were calculated.

PCR amplification of Lys(U)RS and MetRS genes of E. coli

Lys(U)RS gene. E. coli chromosomal DNA (1 μg) was mixed with 0.1 nmole each of the forward and the reverse primers (fp- and rp-KRS, respectively) in a 100 μl reaction consisting of 10 μM Tris.HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 μM dNTPs (B.M.) and 2 units of Taq DNA polymerase (Bangalore Genei, India). The contents were subjected to 25 cycles of successive treatments at 92°C for 1 min, 37°C for 2 min and 65°C for 4 min with a ramp time of 0.01 in a temperature cycler (Coy corporation, USA).

MetRS gene. MetRS gene was amplified using forward and reverse primers (fp- and rp-MRS, respectively). PCR was performed as described above except that 0.1 μg plasmid DNA18 was used as template and the amplification was carried out for 30 cycles at 92°C for 1 min, 25°C for 30 sec and 65°C for 2 min. Ramp time between 92°C to 25°C was 0.01 and 3 min between 25°C to 65°C.

Cloning of PCR products

Purification of PCR products. Aliquots (15 μl) from the PCR reactions were electrophoresed on 0.9% SeaPlaque GTG agarose (Hoeffer Scientific Instruments, Ca, USA) gel in Tris-borate, EDTA buffer (TBE, ref. 19). The Lys(U)RS and the MetRS genes of ~2.0 and 2.2 kb were cut out, rinsed with distilled water for 2 min, made up to ~100 μl with distilled water and melted at 65°C for 5 min. Based on the ethidium bromide fluorescence, there was ~200 and 250 ng DNA in the gel pieces corresponding to Lys(U)RS and MetRS genes, respectively.

In situ UDG reaction on purified PCR products. Reactions (20 μl) containing ~40 ng of the PCR product were carried out as described above except that Tris.HCl (pH 8.0) was used and supplemented with 10 ng (our preparation) or 1 unit (B.M.) of UDG. Reaction mixture was incubated for 1 h at 37°C and transferred to 50°C for 1 h to effect cleavage at the abasic sites.

Preparation of vector. Plasmid, pAC1 (ref. 20) DNA (3 μg) was digested with SacI, treated with calf intestinal alkaline phosphatase (B.M.) according to the supplier's recommendations and electrophoresed on 0.9% SeaPlaque GTG agarose gel in TBE. Gel piece corresponding to linearized vector was cut out, rinsed in distilled water for 2 min, made up to 250 μl with distilled water and melted at 65°C for 5 min.

Ligations and transformations. UDG treated reactions (20 μl) containing ~40 ng DNA were mixed with 10 μl of the vector preparation (~120 ng) (insert to vector ratio on a mole/mole basis was ~1:1.5). The
reaction was supplemented with 4 μl 10 x ligase buffer (660 mM Tris.HCl pH 7.5, 50 mM MgCl₂, 10 mM DTT, 10 mM ATP) and 6 μl distilled water. T4 DNA ligase (0.5 Wiess unit) was added and the reaction mixture kept at 22°C for 12 h. Contents of the reaction were used to transform E. coli TG1 (supE hsdS7r68(dac-proA) F" [traD36proABlacZAM15] using RbCl method⁴. In control experiments performed to determine the efficiency of PCR cloning, Lys(U)RS and MetRS genes were excised from the recombinant plasmids with SacI and electophoresed on low melting agarose. The inserts were prepared and DNA (~40 ng) was used for cloning.

Results

Oligos and their characterization

Oligos and various abbreviations used to denote them are given in Table 1. Figure 1a shows an autoradiograph of 5' [³²P]-end-labelled oligos electrophoresed on 15% polyacrylamide-8 M urea gel. All of the oligos migrate as single bands indicating their purity. Figure 1b shows the results of annealing of 5' [³²P]-end-labelled fp-KRS (lane 1) with anti fp-KRS [duplex1] (lanes 2 and 3). Results show that when complementary oligo was present in 2.5 fold molar excess, all of the 5' [³²P]-end-labelled oligo was driven into a duplex (compare lane 3 with lane 1). Therefore, 2.5 fold molar excess of the complementary oligo was routinely used to prepare double stranded substrates for UDG reactions.

UDG reactions and kinetics of uracil excision

UDG reaction on dUMP containing oligos generates abasic sites at positions occupied by dUMP residues. The abasic sites are sensitive to cleavage under alkaline conditions. As a result of this cleavage two fragments are obtained, one of which ([³²P]-labelled) is detected as a product. In Figure 2, a product of expected size is seen in all reactions. Complete excision of uracil from these oligos fulfills a requirement necessary for their use in the method for cloning PCR amplified DNA.

UDG reactions shown in Figure 2 were performed on single stranded oligos in presence of excess enzyme. However, to develop the cloning procedure shown in Figure 4, it is important that the uracil residues located near the ends of double stranded substrates be excised efficiently. Thus we used duplex1 as a double stranded substrate and analysed the efficiency of uracil release from it under limiting UDG concentration. A time course of UDG reactions is shown in Figure 3. When 3.3 fold higher concentration of UDG was used for duplex1 (i.e. compared to single stranded substrate), intensities of product bands arising out of fp-KRS (lanes 2 to 6) and duplex1 (lanes 8 to 12) were equivalent. This suggested 3 to 4 fold worse excision of uracil from the ends of double stranded substrates. We repeated these kinetics (Materials and Methods) for quantitation and average values calculated from three independent experiments are shown in Table 2. Results show that uracil removal from fp-KRS is retarded by a factor of about 4 when it is in a duplex (duplex1). To see if the reduction in efficiency of uracil release from duplex1 was a result of increased DNA concentration (because 2.5 molar excess of complementary oligo was used to drive fp-KRS into duplex1), we performed kinetics on fp-KRS in presence of 2.5 fold molar excess of anti rp-ungG (an oligo not complementary to fp-KRS). Table 2 (see values for fp-KRS(NC)) suggests that inclusion of the non-complementary oligo results in a slight decrease of uracil excision from fp-KRS. When this is taken into account, efficiency of uracil release from duplex1 is about 3 fold lower than its release from fp-KRS (24% vs 81%).

Cloning of PCR products

PCR products are most commonly cloned by blunt end ligation or by introducing restriction sites in the primers. Both of these methods have certain limitations.⁴ Kinetic analysis of uracil excision shows that UDG excises uracil residues located near the ends of double stranded DNA also. This allowed us to design an efficient method for cloning of PCR products outlined in Figure 4. Sequence of the PCR primers shown in figure 4a is based on the sequences of lysyl-tRNA synthetase [Lys(U)RS] and methionyl-tRNA synthetase [MetRS] genes of E. coli.⁴ The sequence of forward and reverse primers at the 5' end was fixed as 5' AGCU-C-3'. This resulted in one or two mismatches with the respective gene sequences (Figure 4a). But the location of dUMP in each of the primers is such that UDG treatment of PCR products results in an overhang, identical to the one generated by SacI. As the cleavage of the DNA chain at abasic sites under alkaline pH after the UDG reaction results into a 5'-phosphate on the next nucleotide, the PCR products can be directly cloned into a dephosphorylated vector.

Figure 5b shows PCR amplifications of the Lys(U)RS and the MetRS genes of E. coli. The PCR products were cut out of a low melting agarose gel and cloned (Materials and Methods) into a low copy vector, pACI. Presence of the inserts in the clones was confirmed by digestion of the plasmid mini-preparations with HindIII. A representative photograph of an agarose gel stained with ethidium bromide is shown in Figure 6a. A HindIII
**Figure 1.** Electrophoresis of 5'[^32]P-end-labelled oligos on: (a) denaturing (15% polyacrylamide-8 M urea) and (b) non-denaturing (12% polyacrylamide) gels. Names of oligos are shown on top of each lane. Molar ratios of anti fp-KRS to form duplex1 from fp-KRS are shown in panel b.

**Figure 2.** Analysis of UDG reactions on a 15% polyacrylamide-8 M urea gel. Reactions were performed with (+) or without (-) UDG. Names of the oligos are shown on top of the lanes. Substrate (S) and product (P) are indicated.

**Figure 3.** Kinetics of uracil release from fp-KRS and duplex1. Autoradiograph of a representative gel showing release of uracil from single stranded fp-KRS (lanes 1 to 6) and its double stranded form, duplex1 (lanes 7 to 12). Lanes 1 and 7 are controls where no enzyme was added and was incubated for 10 min. Reaction mixtures of other reactions are shown above each lane. Amounts of UDG used for this set of reactions were 0.75 µg for fp-KRS and 2.5 µg for duplex1.

Site is present within the Kan' gene of the vector (not shown), so a single band of ~6.6 kb (lanes 1 to 6) shows the presence of Lys(U)RS gene within these clones. As the MetRS gene itself contains an asymmetrically located HindIII site, fragments of 3.7 and 3.1 or...
Table 2. Kinetics of uracil excision

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Efficiency (relative to fp-KRS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fp-KRS</td>
<td>100</td>
</tr>
<tr>
<td>Duplex1</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>fp-KRS(NC)</td>
<td>81 ± 4</td>
</tr>
</tbody>
</table>

Values have been shown to the nearest complete number. Values are
average of three independent experiments Duplex1 was formed by
annealing fp-KRS with 2.5 molar excess of anti fp-KRS, fp-KRS(NC) denote
fp-KRS mixed with 2.5 molar excess of anti fp-ungG.

Figure 4. Outline of cloning strategy

3.6 and 3.2 kb (depending on the orientation of the
insert) in lanes 10–13 show the presence of the insert
in these clones. A single fragment of ~4.6 kb in lanes
8 and 9 shows that no insert was cloned within these
plasmids.

Release of DNA fragments of ~2.0 kb from Lys(U)RS
and ~2.2 kb from MetRS clones (Figure 6b, lanes 1 to
6 and 10 to 13, respectively) upon digestion with SacI
confirms that the SacI site was generated at the junctions.

For comparison of cloning efficiencies, we repeated
the UDG cloning experiment and as a control, we also
cloned the Lys(U)RS and the MetRS genes that were
excised as SacI restriction fragments from the recombi-
nants obtained in the first attempt. Analysis of a larger
number of plasmid mini-preparations is summarized in
Table 3. The efficiencies of cloning of the PCR products
are about the same as those obtained for subcloning of
the respective restriction fragments. In addition, SDS-
PAGE analysis of the cell free extracts from the recombi-
nant clones also showed overexpression of the
respective amino acyl-tRNA synthetases (data not
shown).

As the above experiments were done with our prepara-
tion of UDG, we repeated the cloning of the PCR
products of Lys(U)RS gene using the commercially
available UDG (B.M.). Table 3 shows that the cloning
efficiency remained the same.

Discussion

Our data on kinetics of uracil release from oligomeric
DNAs show that UDG uses single stranded substrates
~3 fold better than the double stranded substrates. This
is similar to what has earlier been shown for natural
DNA substrates. More importantly, our data show that
UDG excises uracil residues from the ends of double
stranded substrates albeit at a slightly reduced rate when
compared to its excision from single stranded substrates
(Figure 3, Table 2). In addition to this, the method that
we have developed for cloning of the PCR products
has been a result of several other important observations.
For example, the UDG is active even in low melting
agarose gels, and to effect a complete cleavage at the
abasic sites, harsher conditions (NaOH/boiling) are not
necessary (data not shown). It was therefore possible
to cut out the PCR products from the low melting
agarose gel, treat with UDG, effect abasic site cleavage
and ligate into the vector, all in one tube; and use the
contents directly for transformation. Agarose gel
electrophoresis step increases overall efficiency as it
results in elimination of the unused primers (which
interfere in the UDG reaction) and other undesired
products that arise during PCR amplifications, from
the samples. Further, simultaneous elimination of the dNTPs
during agarose gel electrophoresis also increases the
ligation efficiency.

Non-templated addition of the single nucleotides to
the 3′ ends of PCR products by the terminal transferase
activity of Taq DNA polymerase drastically reduces
the efficiency of cloning by blunt end ligation. In our
method we employed use of ‘sticky’ ends to increase
the efficiency of ligation. The cloning of PCR products
using this method was as efficient as that of equivalent
restriction fragments. Therefore, it is likely that single
nucleotide extensions to the 3′ ends of PCR products
do not significantly affect ligation of the 3′[OH] of the
vector overhang to the 5′[PO4] of the PCR product
(insert). Further, the inserts from all of the recombinant
clones could be excised by SacI suggesting that the 3′
ends of the PCR products were correctly repaired in vivo.

Recently, Rashchien et al. also described a method
for cloning of PCR products which utilizes UDG. The
UDG cloning procedure described by us, is novel in
that: (i) dUMP is placed at a key position in the primers
which eliminates the need for longer primers containing
multiple dUMP residues, (ii) no specialized vectors are
needed for cloning, and (iii) the procedure does not
result in addition of the repeat sequences to the ends
(i) Lys(U)RS

\[ \text{5'}-\text{agcucgccggaatgatacagag} \rightarrow \text{Lys(U)RS} \rightarrow \text{AACCAcGGGTCCACCACTACAGTCCCTAAAA-3'} \]

\[ \text{5'}-\text{CTGTTAGCCAGGCGGAGGAATGATACAGGAG} \rightarrow \text{tggtccacag} \rightarrow \text{CgatCgatUcga-5'} \]

(ii) MetRS

\[ \text{5'}-\text{agctctacgtacag} \rightarrow \text{MetRS} \rightarrow \text{AAAATTACAGGATGCAGAATGACACATTTA-3'} \]

\[ \text{5'}-\text{ACCACCTGACCTGATAGTTACCCTGAGAATAT} \rightarrow \text{tttaattgctcctacggttacUcga-5'} \]

Figure 5. a. Design of the PCR primers. Relevant sequences of lysyl and methionyl-tRNA synthetase genes [Lys(U)RS, and MetRS, respectively] of E. coli are shown. Forward and reverse primers, respectively, are shown above or below the main sequence. Location of the dUMP residue in the primers is shown in block letter (U) and asterisks show positions where primer sequences mismatch with those of the genes. b. Agarose (1%) gel electrophoresis of PCR products. Lane (M) shows DNA size markers obtained by HindIII and HinfIId digestion of lambda DNA. Approximate sizes in kb are 4.6, 3.8, 3.2, 2.9, 2.2, 2.1, 2.0, 1.9, 1.7, 1.6, etc.

Figure 6. Agarose (1%) gels showing digestion of planned transcript preparations with HindIII a or SacI b. Clones of Lys(U)RS in lanes 1 to 6 are labelled as KRS whereas those of MetRS are labelled as MRS (lanes 8 to 11). Lane 7 shows DNA size markers described in legend to Figure 5(b).
Table 3. Cloning of PCR products

<table>
<thead>
<tr>
<th>Source of insert</th>
<th>Clones obtained</th>
<th>Clones analysed</th>
<th>Clones with insert</th>
<th>Clones with SacI excisable insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>MetRS [SacI]</td>
<td>65</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>MetRS [PCR, UDG]</td>
<td>28</td>
<td>10</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>LytURS [SacI]</td>
<td>79</td>
<td>10</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>LytURS [PCR, UDG]</td>
<td>94</td>
<td>10</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>*LytURS [SacI]</td>
<td>39</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>*LytURS [PCR, UDG (B M)]</td>
<td>31</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Cloning efficiencies of PCR products. SacI shown within brackets indicates that inserts were obtained by SacI digestion [PCR, UDG] indicates that the insert was generated by treatment of the PCR products with UDG (our preparation) [UDG, (B M)] shows use of commercial UDG (B M). Asterisks indicate that these two clonings were done in a separate experiment.

of the desired DNA fragment. This may be an important consideration while cloning into the expression vectors. Directional cloning by the method reported here should be possible by designing PCR primers which will generate an AatII overhang at one end and the SacI on the other. In future, it should also be possible to incorporate other modified bases in the primers and use appropriate DNA glycosylases to allow cloning within the sites other than SacI or AatII. Since we observed a high efficiency of cloning, this general method of using DNA glycosylases for cloning and; the properties and kinetics of UDG described here will be beneficial in its effective use for a wide variety of genetic engineering applications.


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Faunal affinity and zoogeography of Recent marine ostracoda from Karwar, West Coast of India

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The occurrence of 80 Recent marine ostracode taxa around Karwar, West Coast of India, has been recorded. Their ecology, affinity and zoogeographic distribution are also discussed briefly.

Recent ostracodes, also called proxy indicators or seed shrimps, have gained much importance in the recent years in view of their environmental significance and application in search for oil and gas in the offshore regions of India. Studies of these bivalved microcrustaceans are, therefore, important for a better understanding of their fossil counterparts of these regions.

Besides, they also serve as indicator organisms in pollution studies.

A perusal of literature on Recent ostracodes from West Coast of India reveals that little attention has been paid to their taxonomic, ecological and zoogeographic studies. Most of the publications are preliminary in nature and/or make only a casual reference to them. The only important studies report 13 taxa (2 new genera, 1 new subgenus, 1 new species) from the Anjiv Island, followed by the record number (56) of species (1 new genus, 1 new subgenus and 16 new species) from Mandvi Beach in Gujarat and 34 species (including 4 new species) from Kerala Coast. The present investigation is based on systematic studies of Recent ostracoda around Karwar, North Karnataka Coast, facing the Arabian Sea (Figure 1). In all 228 sediment samples, an average of 11 samples from each of the fourteen beaches and two deltas and one each from forty-eight (1-48) inner-shore stations (19-71 m depth) were collected for micropalaeontological studies. These

![Figure 1](currentsciencejournals.com/1994/78/10/715/1994_78_10_715.png)