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

N. Vinay Kumar and U. Varshney
Centre for Genetic Engineering, Indian Institute of Science, Bangalore 560 012, India

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[1-3]. Using dUMP containing viral DNAs, it was shown that UDGs use single stranded substrates more efficiently than the double stranded substrates[4-7]. 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[8]. Recently, UDG has become an important enzyme in DNA research[9-14]. 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*[4,15]. 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 5′[^{32}P]-end-labelling was performed as described in ref. 8, 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. The ^{32}P-labelled oligos were purified by chromatography on Sephadex G-50 minicolumns.

Formation of double stranded oligos

5′[^{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 1. List of the oligos

Oligo	Size (Nt.)	Sequence
fp-MRS	25	d(agcUcatagtttacctgaagaatat)
rp-MRS	26	d(agcUcattctgcatccctgtaatttt)
fp-KRS	27	d(agcUcgccgacgaatatgatacaggag)
rp-KRS	27	d(agcUctagttgagtggacccgctggtt)
anti fp-KRS	27	d(cacctgtatcatattcgtcggcgagct)
anti rp-ungG	19	d(ggggaaatgccgggtggca)

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

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 $MgCl_2$) (ref. 16) prior to use.

Uracil DNA glycosylase (UDG) reactions

Standard conditions. Oligos (10 pmoles) 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 UDG 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 (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 cyanolFF and bromophenol blue, and 1 mM Na_2EDTA). Aliquots (5 µl) were analysed on 15% polyacrylamide-8 M urea gels[17] of 0.8 mm thickness and exposed to Indu X-ray films (Hindustan Photo Films, India) with or without hyperscreens (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′[^{32}P]-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 mM Tris.HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 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 DNA[18] 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 × ligase buffer (660 mM Tris.HCl pH 7.5, 50 mM $MgCl_2$, 10 mM DTT, 10mM 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 hsd*Δ5*thi*Δ(*lac-proAB*)) F′[*traD*36*proAB*$^+$*lacI*^{q}*lacZ*ΔM15] using RbCl method[21]. 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 electrophoresed 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 1*a* shows an autoradiograph of 5′ [^{32}P]-end-labelled oligos electrophoresed on 15% polyacrylamide-8 M urea gel. All of the oligos migrate as single bands indicating their purity. Figure 1*b* shows the results of annealing of 5′ [^{32}P]-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′ [^{32}P]-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 ([^{32}P]- 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[22]. 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 5*a* is based on the sequences of lysyl–tRNA synthetase [*Lys(U)RS*] and methionyl-tRNA synthetase [*MetRS*] genes of *E. coli*[23,24]. The sequence of forward and reverse primers at the 5′ end was fixed as 5′ AGCUC—3′. This resulted in one or two mismatches with the respective gene sequences (Figure 5*a*). 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 5*b* 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, pAC1. 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 6*a*. A HindIII

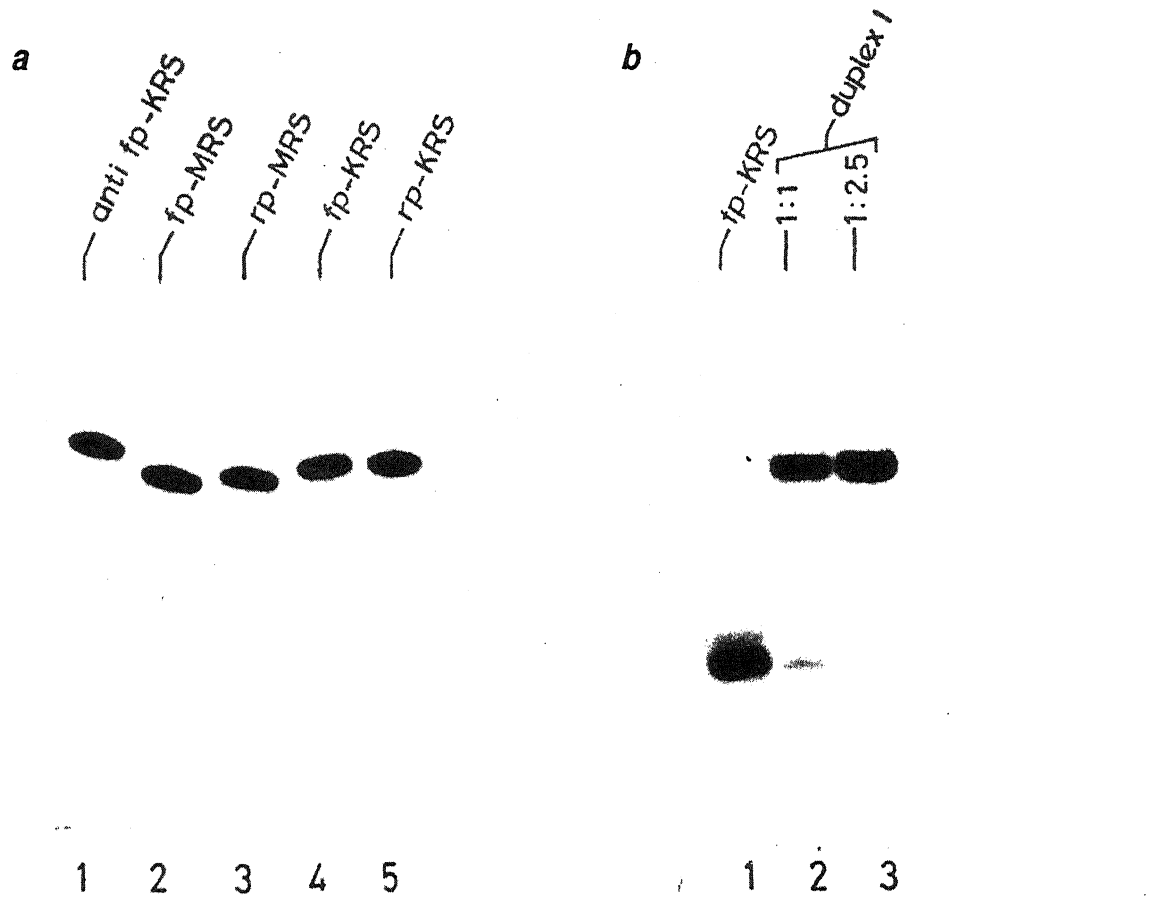

Figure 1. Electrophoresis of 5′ [^{32}P]-end-labelled oligos on: (*a*) denaturing (15% polyacrylamide-8 M urea) and (*b*) non-denaturing (12% polyacrylamide) gels. Names of oligos are shown on top of each lane. Molar ratios of anti fp-KRS to form duplex1 from fp-KRS are shown in panel *b*.

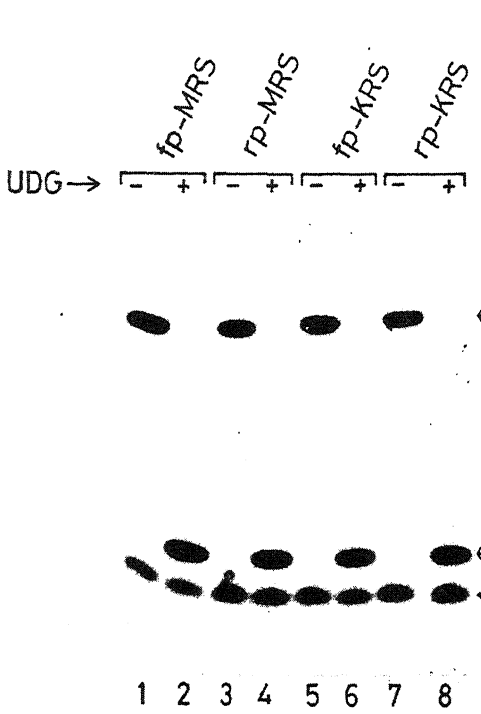

Figure 2. Analysis of UDG reactions on a 15% polyacrylamide-8 M urea gel. Reactions were performed with (+) or without (−) UDG. Names of the oligos are shown on top of the lanes. Substrate (S) and product (P) are indicated.

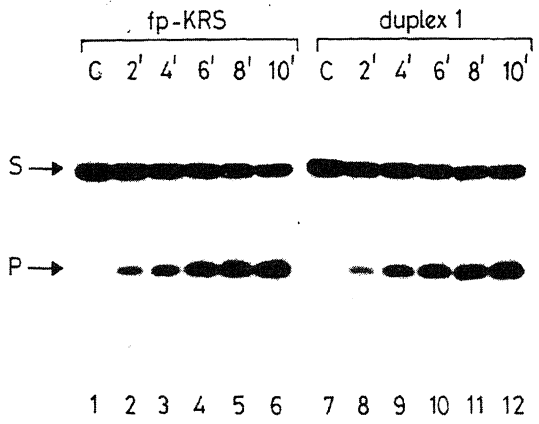

Figure 3. Kinetics of uracil release from fp-KRS and duplex1. Autoradiograph of a representative gel showing release of uracil from single stranded fp-KRS (lanes 1 to 6) and its double stranded form, duplex1 (lanes 7 to 12). Lanes 1 and 7 are controls where no enzyme was added and was incubated for 10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

site is present within the *Kan*^{r} 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

Substrate	% Efficiency (relative to fp-KRS)
fp-KRS	100
Duplex1	24 ± 5
fp-KRS(NC)	81 ± 4

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) denotes fp-KRS mixed with 2.5 molar excess of anti rp-ungG.

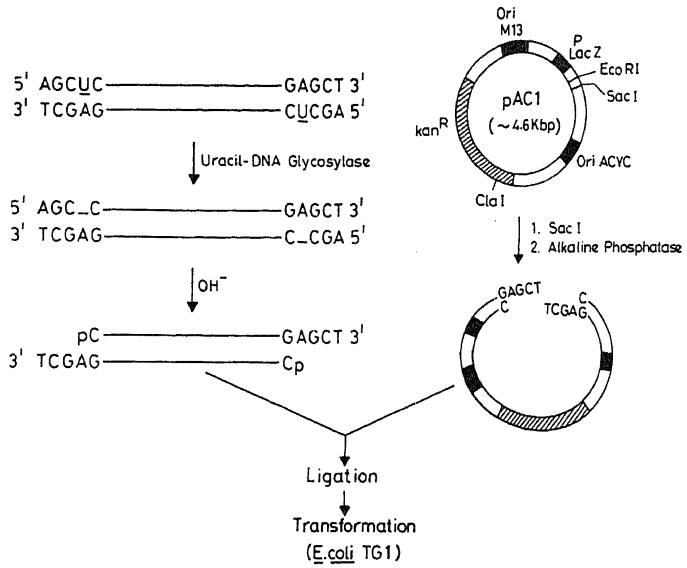

Figure 4. Outlines of cloning strategy.

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 6*b*, 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 recombinants 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 recombinant clones also showed overexpression of the respective amino acyl-tRNA synthetases (data not shown).

As the above experiments were done with our preparation 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[4,6]. 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[25] drastically reduces the efficiency of cloning by blunt end ligation[22]. 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′ [PO_4] 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, Rashtchian *et al.*[13] 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

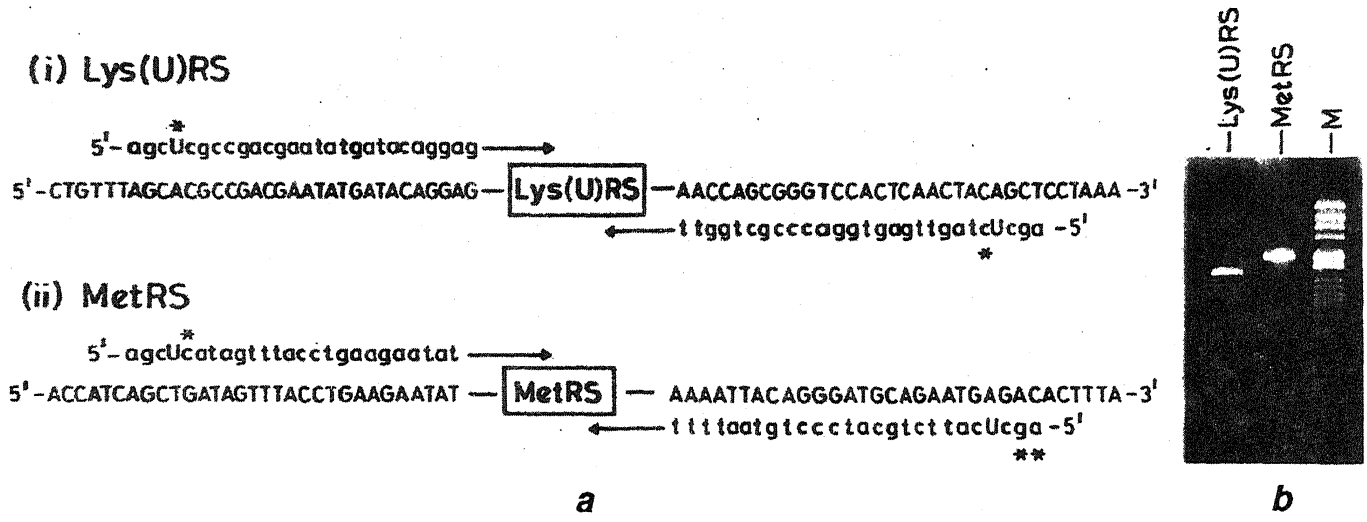

Figure 5. *a*, Design of the PCR primers. Relevant sequences of lysyl and methionyl-tRNA synthetase genes [*Lys(U)RS*, and *MetRS*, respectively] of *E. coli* are shown. Forward and reverse primers, respectively are shown above or below of the main sequence. Location of the dUMP residue in the primers is shown in block letter (U) and asterisks show positions where primer sequences mismatched with those of the genes. *b*, Agarose (1%) gel electrophoresis of PCR products. Lane (M) shows DNA size markers obtained by HindII and HindIII 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.

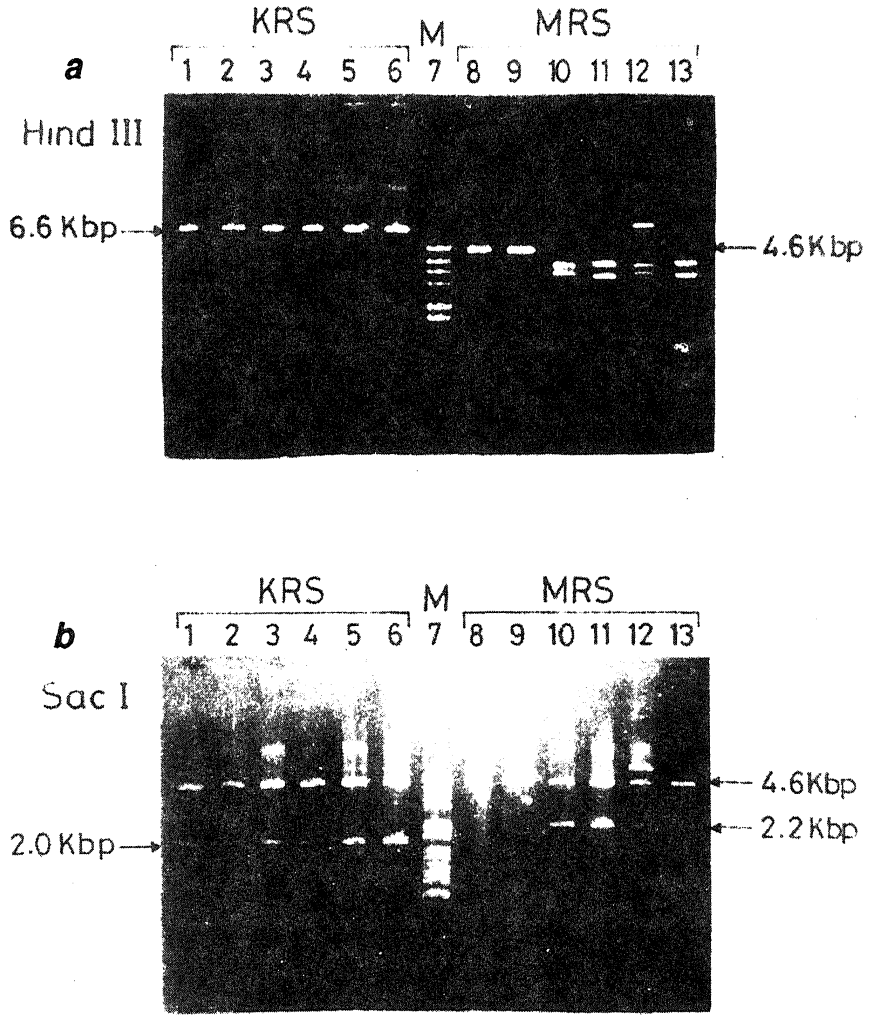

Figure 6. Agarose (1%) gels showing digestion of plasmid mini-preparations with HindIII *a* or SacI *b*. Clones of Lys(U)RS in lanes 1 to 6 are labelled as KRS whereas those of MetRS are labelled as MRS (lanes 8 to 13). Lane 7 shows DNA size markers described in legend to Figure 5(*b*).

Table 3. Cloning of PCR products

Source of insert	Clones obtained	Clones analysed	Clones with insert	Clones with SacI excisable insert
MetRS [SacI]	65	10	8	8
MetRS [PCR, UDG]	28	10	6	6
Lys(U)RS [SacI]	79	10	7	7
Lys(U)RS [PCR, UDG]	94	10	7	7
*Lys(U)RS (SacI)	39	10	9	9
*Lys(U)RS [PCR, UDG (B.M.)]	31	10	8	8

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[4] 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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