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Generation of Cohesive Ends on PCR Products by UDG-mediated Excision of dU, and Application for Cloning into Restriction Digest-linearized Vectors

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We have investigated the use of dU excision by uracil *N*-glycosylase (UDG) to create cohesive ends on PCR fragments "mimicking" those generated by restriction enzymes. The feasibility of this approach for directional and nondirectional cloning using cohesive ends mimicking *SacI* or *PstI* ends is demonstrated by the subcloning of a 383 to 388-bp fragment of bovine basic fibroblast growth factor into restriction enzyme-linearized pT7T318U. UDG-mediated cohesive ends imperfectly matched to *PstI*-generated vector ends gave reasonable cloning efficiency and accuracy, suggesting that the approach may be extended to mimicry of other restriction enzymes producing 3' overhangs. The rapid and specific excision of dU by UDG (within 30 min at 37°C) has several potential advantages over the use of restriction site-modified primers, including the avoidance of restriction cleavage at internal sites within the PCR product. Also, following ligation, the approach described may be used to prevent subsequent cleavage of the joined DNA segments by the restriction enzyme, that is, by not recreating the restriction enzyme recognition sequence at the junction, which may find application in gene engineering. By adapting the approach to use dU-containing linkers or "vectorettes," the approach may be used for cloning unknown sequences (e.g., by cDNA or genomic li-

brary construction) or for mimicking 5' overhang cohesive ends on PCR fragments.

Several PCR methods have been described for nondirectional and directional cloning (or subcloning) of PCR products as blunt-ended or cohesive-ended fragments.⁽¹⁻⁶⁾ Blunt-ended cloning is generally of low efficiency, despite the use of fill-in reactions (e.g., using Klenow),⁽⁶⁾ although higher efficiencies have been obtained if PCR products are subjected to proteinase K digestion (to remove bound *Taq* polymerase) prior to ligation.⁽⁷⁾ Cohesive end cloning of PCR products generated by the inclusion of restriction enzyme recognition sequences in the amplimers gives higher cloning efficiencies,⁽⁸⁾ although the position and nature of the recognition sequences within the amplimers may influence both amplification efficiency and specificity, and subsequent restriction digest efficiency.⁽²⁾ Furthermore, cleavage at nonamplimer restriction sites may prevent cloning of full-length PCR products. More recently, an efficient cloning procedure has been developed whereby dT residues are replaced by dU within the amplimers and cohesive ends are generated by dU excision using uracil DNA-glycosylase (UDG).^(9,10)

Here, we report an alternative strategy on the basis of inclusion of a single dU residue within the 5' proximal portion

of PCR amplimers which, following amplification, allows specific digestion of the PCR products with UDG to generate 3' overhang cohesive ends "mimicking" those generated by restriction enzymes. These cohesive ends are then capable of annealing to restriction enzyme-generated cohesive ends for directional and nondirectional cloning of PCR products. The feasibility of the approach is demonstrated for the subcloning of a protein-encoding fragment of a bovine basic fibroblast growth factor (FGF) cDNA clone⁽¹¹⁾ by mimicry of *SacI* and/or *PstI* cohesive ends. The data demonstrate that this approach can be used successfully for PCR cloning into the polylinkers of existing vectors with roughly equal efficiency to that obtained using restriction enzyme-based approaches. Incorporating dU at differing positions within the amplimers, giving more or less compatibility between PCR product and linearized vector cohesive ends, also highlights parameters that may affect the overall utility of this strategy; for example, the numbers of restriction enzyme sites that can be successfully mimicked. The approach offers several technical advantages and may be used to recreate or abolish the mimicked restriction site; we have termed the latter invisible mending.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides for the amplification of a 383- to 388-bp fragment of bovine

basic FGF were designed using published cDNA sequence data⁽¹¹⁾ and synthesized using standard chemistries, by Dr. J.E. Fox (Alta Bioscience, Birmingham University, Department of Biochemistry, Birmingham, UK). Deoxyuracil triphosphate (dUTP) analogs were purchased from Cruachem (Glasgow, UK). The sequence and nomenclature given to the primers are shown in Figure 1, which also shows the nature of cohesive ends expected following digestion with *Bgl*III, *Pst*I, or UDG.

To act as tracers to monitor digestion efficiency, forward (5') primers were 5' end-labeled using [³²P]dCTP using a kit supplied by Amersham International plc (Buckinghamshire, UK).

PCR amplifications

PCR reactions were performed in a total volume of 50 μ l [reaction buffer = 10

mm Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.001% gelatin] containing 0.2 mM of each dNTP, 1 μ M of each primer, and 0.1 ng of template DNA. A 1.4-kb (bases 1–1401) portion of a bovine basic FGF cDNA insert excised from the plasmid pBR322 using *Eco*RI⁽¹¹⁾ (the plasmid was a kind gift from Dr. Andrew Baird, Whittier Institute, LaJolla, CA) was used as original template. After initial denaturation at 98°C for 7 min, 2.5 units of *Taq* DNA polymerase (Advanced Biotechnologies Ltd., London, UK) was added, followed by 30 amplification cycles consisting of 96°C for 2 min, 45°C for 1 min, and 72°C for 2 min (although higher annealing temperatures have also been used).

Enzyme digestion of PCR products

Directly following amplification, 1 unit of UDG (Perkin-Elmer Cetus, ILS, London, UK) was added and the reaction

mixtures incubated at 37°C for 30 min. The weakened DNA backbone was cleaved either by heat denaturation (98°C for 15 min) or by the addition of 0.2 volumes of 1 M NaOH, incubation at 37°C for 10 min followed by neutralization with 0.1 volume of 3 M NaOAc (pH 5.5). Cleaved PCR products were then purified using a Magic PCR prep kit (Promega, Southampton, UK) followed by ethanol precipitation. All restriction enzyme (30 units) digests were performed on purified PCR products at 37°C for 1 hr in 30 μ l of reaction buffer.

Concentrations of purified PCR products and linearized vector (see below) were determined using a sensitive fluorimetric assay.⁽¹²⁾

Cloning of PCR products into pT7T318U

Two-micrograms samples of pT7T318U

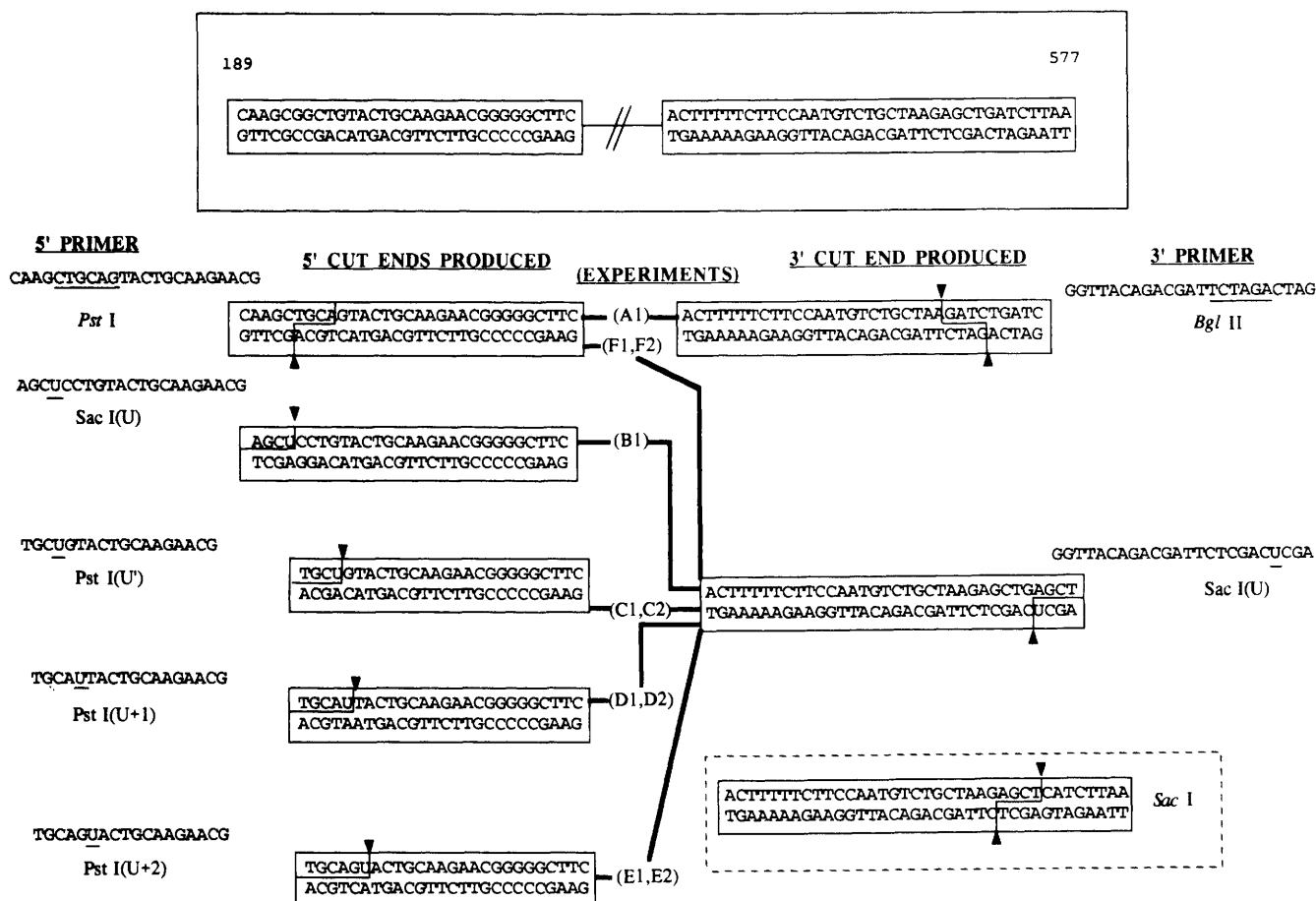


FIGURE 1 Schematic representation showing the amplimer sequences used to amplify a 385- to 388-bp fragment (dependent on position of binding of amplimers) of plasmid DNA containing a cDNA encoding bovine basic FGF *top*. The positions of cleavage with the respective restriction endonuclease or UDG are indicated with arrow heads, and the cohesive ends generated are shown. The equivalent ends generated using a *Sac*I-modified primer are shown at *bottom* (broken lines). Primers containing dU mimics are denoted by the comparable restriction enzyme name (roman type) followed by (U), with (U'), (U + 1), (U + 2) distinguished among the imperfect mimics resulting in a mismatch, a one-, or a two-base gap between overhangs, respectively.

(Pharmacia/LKB, Milton Keynes, UK) were digested in 50 μ l of the appropriate buffer containing 20 units of restriction enzymes (*Bam*HI, *Pst*I, *Sac*I) for 1 hr at 37°C, and selected preparations incubated with 2.5 units of calf intestinal alkaline phosphatase (CIP, Pharmacia/LKB) in 6 μ l buffer [1 M Tris (pH 8.0), 1 μ l 10% (wt/vol) SDS] for 2 hr at 37°C. Both CIP-treated and untreated linearized vector samples were purified by Magic DNA purification kits (Promega) and ethanol precipitated.

Ligation of purified cohesive-ended PCR products and linearized pT7T318U vector (Pharmacia/LKB, Milton Keynes, UK), and subsequent transformation of *Escherichia coli* XL1 blue were performed using established protocols. In all cases, roughly equimolar amounts of purified PCR products (10 ng) and *Bam*HI, *Pst*I, or *Sac*I linearized vector (50 ng) were used in each cloning experiment. Transformants were selected by ampicillin resistance.

Recombinant colonies were analyzed by insert size using restriction digestion and/or PCR amplification. Essentially recombinant colonies were dispersed in 50 μ l of deionized water, and 25 μ l was then used to inoculate LB media cultures while the remaining 25 μ l was boiled (100°C) for 5 min and 1 μ l used as template in PCR amplification under the conditions described above. Plasmids were also prepared from 1.5 ml of overnight cultures using standard alkaline lysis miniprep procedures and subject to restriction enzyme digest analysis.

RESULTS

Amplification and digestion of PCR products

All of the primer combinations indicated in Figure 1 consistently, and with roughly equivalent efficiency, amplified a predominant PCR product of the expected size (see Fig. 1) from a cDNA clone encoding bovine basic FGF (see Fig. 2A). The authenticity of the product was confirmed in all cases by restriction mapping (data not shown). Because the 5' proximal position of the dU required for cohesive end mimicry may have posed problems in terms of accessibility to excision by UDG, we monitored the loss of radioactivity from tracer amounts of 5' ³²P-end-labeled primers incorporated within the PCR products following

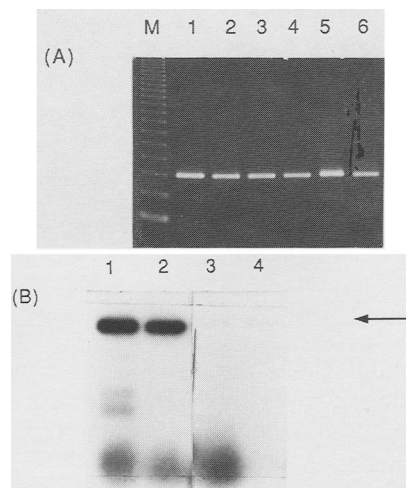


FIGURE 2 Amplification and digestion of PCR products. (A) Electrophoretic separation [1% (wt/vol) agarose] of PCR products generated using primer combinations indicated in Table 1 (lanes 1–6 correspond to experiments A–F, respectively). The specific amplification products (ranging between 383 and 388 bp) are shown. (B) Autoradiograph (overnight exposure) of PCR products generated using tracer amounts of ³²P-labeled (5' end) forward (5') amplimers before (lanes 1–2) and following digestion with *Pst* I (lane 3) or UDG (lane 4). The arrow marks the position of 383- to 388-bp amplification products.

incubation with UDG or *Pst*I. Figure 2B shows an example of the efficiency of digestion of the PCR products with *Pst*I (1 hr, 37 °C) or UDG (30 min, 37 °C) followed by heat denaturation. Almost equivalent amounts of 5' ³²P-labeled tracer amplimer sequences have been removed in both cases. Figure 2B also shows how unincorporated dU-containing amplimers are degraded during incubation with UDG while unincorporated restriction-site modified amplimers remain intact (as evidenced by the diffuse low-molecular-weight bands).

Cloning of UDG-generated cohesive-ended fragments

PCR products digested with restriction enzymes and/or UDG were investigated for their relative capacity to be directionally and nondirectionally cloned into restriction enzyme-linearized pT7T318U. Initial cloning experiments comparing efficiencies using heat or alkali treatment demonstrated that alkali treatment gave greater cloning efficiencies following UDG excision of dU (data not

shown), attributable to the covalent cleavage at abasic sites effectively removing 5' nicked sequences from the PCR products. All data shown in Tables 1 and 2 derive from experiments performed using alkali treatment protocols following dU excision by UDG. The cloning efficiencies obtained using restriction enzyme or dU excision to generate 3' overhangs are summarized in Table 1, which also shows the number of true recombinant colonies, as determined by PCR amplification and/or restriction enzyme digestion of plasmid preparations. Comparable numbers of true recombinants were recovered following directional insertion of PCR fragments generated using *Pst*I + *Bgl*II-modified and *Pst*I(U+1) + *Sac*I(U)-modified primers. An expectedly higher recovery of true recombinant colonies was obtained following nondirectional cloning of PCR products generated using *Sac*I(U) primers. Directional cloning of both *Sac*I(U) + *Pst*I(U') and *Sac*I(U) + *Pst*I(U+2) primer products gave a comparable number of recombinants to that obtained using UDG *Sac*I products alone. However, only 25% of recombinants generated from *Pst*I(U+2) + *Sac*I(U) primer products contained correct inserts (see Table 1, Experiment E1). The efficiency of cloning, as judged by the number of recombinants, was, however, significantly lowered in the case of all PCR products generated using *Pst*I(U) mimics if the vector ends were pretreated with CIP. Significantly, directional cloning of cohesive-ended PCR fragments generated using *Pst*I and *Sac*I(U)-modified primers demonstrated an increased number and frequency of true colonies and was unaffected by pretreating vector ends with CIP (See Table 1, Experiments F1 and F2).

Recreating restriction sites following ligation of UDG-generated cohesive ends

As can be seen from Table 2 the ability to recreate restriction sites following ligation of UDG-generated cohesive ends to restriction enzyme-generated cohesive ends is dependent on the degree of compatibility. Thus, 100% of recombinants generated using *Pst*I- and/or *Sac*I(U)-modified primer products were capable of being recut using the respective restriction endonuclease. In contrast, we could detect no digestion by *Pst*I of nine

TABLE 1 Summary of Relative Cloning Efficiencies Obtained in Cloning Experiments by Use of PCR Fragments with Cohesive Ends Generated Using Restriction Endonuclease (*Bgl*III and *Pst*I) or UDG Digestion of Amplimers

Experiment	Amplimers used ^a		Number of colonies ^b
	forward (5')	reverse (3')	
A1	<i>Pst</i> I	<i>Bgl</i> III	252 (8/10) ^c
B1	<i>Sac</i> I(U)	<i>Sac</i> I(U)	778 (9/10) ^c
C1	<i>Pst</i> I(U')	<i>Sac</i> I(U)	662 (9/10) ^c
C2 ^d	<i>Pst</i> I(U')	<i>Sac</i> I(U)	25 NT ^e
D1	<i>Pst</i> I(U+1)	<i>Sac</i> I(U)	288 (9/10) ^c
D2 ^d	<i>Pst</i> I(U+1)	<i>Sac</i> I(U)	51 NT ^e
E1	<i>Pst</i> I(U+2)	<i>Sac</i> I(U)	542 (5/20) ^c
E2 ^d	<i>Pst</i> I(U+2)	<i>Sac</i> I(U)	114 NT ^e
F1	<i>Pst</i> I	<i>Sac</i> I(U)	2044 (10/10) ^c
F2 ^d	<i>Pst</i> I	<i>Sac</i> I(U)	1716 NT ^e
Control experiments using vector alone cut with			
	<i>Sac</i> I		46
	<i>Sac</i> I and <i>Bam</i> HI		40
	<i>Pst</i> I and <i>Sac</i> I		48
	<i>Pst</i> I and <i>Bam</i> HI		28
	<i>Pst</i> I and <i>Sac</i> I (followed by CIP treatment)		36
	Uncut vector alone (1 ng)		1124

^aSee Fig. 1 legend for amplimer nomenclature.

^bTotal number of colonies following overnight incubation.

^cFigures in parentheses indicate number of colonies tested positive for correct inserts by PCR amplification from plasmid minipreps.

^dIn these experiments, cut vector ends were CIP treated before insertion and ligation of PCR product.

^e(NT) Not tested.

plasmid preparations isolated from recombinants harboring PCR fragments directionally cloned using 3' cohesive ends generated from primer *Pst*I(U+1) by UDG excision of dU. In contrast, 33% (three of nine) recombinants generated using the *Pst*I(U') primer products were recognized and cleaved by *Pst*I, whereas 100% of recombinants (five of five) harboring PCR products generate using *Pst*I(U+2)-modified primers were cut by *Pst*I. It should be noted, however, that the percentage of true recombinants re-

covered using this amplimer was only 25%; see Table 1, Experiment E1.

DISCUSSION

The ability of UDG to specifically cleave at positions where dU has been incorporated during PCR has been utilized for cloning,^(9,10) sequencing,⁽¹³⁾ mutagenesis,⁽¹⁴⁾ and prevention of carryover contamination.^(15,16) Here, we describe a cloning strategy whereby excision of a single dU appropriately positioned in

TABLE 2 Summary of Results Obtained when Recombinants Harboring Correct Inserts Were Digested with Either *Pst*I or *Sac*I

Amplimer pairs (5') (3')	Experiment	Number of recombinants cut with	
		<i>Pst</i> I	<i>Sac</i> I
<i>Pst</i> I + <i>Bgl</i> III	A1	10/10	NA ^a
<i>Sac</i> I(U) + <i>Sac</i> I(U)	B1	NA ^a	10/10
<i>Pst</i> I(U') + <i>Sac</i> I(U)	C1	3/9	9/9
<i>Pst</i> I(U+1) + <i>Sac</i> I(U)	D1	0/9	9/9
<i>Pst</i> I(U+2) + <i>Sac</i> I(U)	E1	5/5	5/5
<i>Pst</i> I + <i>Sac</i> I(U)	F1	10/10	10/10

^a(NA) Not applicable.

the amplimer can be used to generate cohesive ends suitable for cloning into restriction enzyme-linearized vectors.

At the outset of this study we recognized that the degree of compatibility of cohesive ends generated by UDG or by restriction enzymes may be a significant factor limiting cloning efficiency. We therefore chose to mimic *Sac*I and *Pst*I cohesive ends, with the former representing the situation where the incorporation of dU has no effect on the original sequence (e.g., merely represents substitution of dT for dU) and the latter allowing study of cohesive ends varying in the degree of compatibility to a *Pst*I-generated cohesive end (see Fig. 1). The data in Table 1 show that directional cloning of the PCR products can be achieved using both perfectly and imperfectly compatible overhangs. Other restriction enzymes (e.g., *Bgl*I, *Kpn*I, *Hha*I, *Hae*II, *Pvu*I, *Sac*II) that generate 3' overhangs may therefore be amenable to mimicry using dU excision by UDG, even though gaps or overlaps are introduced. The data do, however, demonstrate that imperfectly mimicked *Pst*I cohesive ends may not be efficiently ligated if the vector ends are treated with CIP.

Our preliminary investigations suggest that there are several ways in which the UDG excision approach outlined here may have advantages over traditional methods using restriction enzyme-generated cohesive ends. For example, restriction enzyme cleavage of PCR fragments generated using restriction site-modified primers may also cleave the PCR product at internal (non-amplimer) sites. This potential problem is eliminated using the approach outlined here. It is also possible that dU containing restriction site mimic linkers may be developed which, followed dU excision by UDG could be used to avoid this phenomenon during non-PCR-based cloning, for example, of unknown sequences by genomic or cDNA library construction. Development of vector-ettes⁽¹⁷⁾ containing dU may also allow this approach to be used to mimic restriction enzyme-generated cohesive ends with 5' overhangs.

Many restriction enzymes require that the recognition sequence be located centrally or 3' proximal within the primer for effective digestion which, in some instances, may reduce efficiency and/or specificity of the PCR amplification. The position of the dU at the

5'proximal end of the amplified DNA presents no such recognition problem, being readily excised by UDG (see Fig. 2). We have recently used this approach to clone human apolipoprotein genomic segments ranging in size from 1.7–4.2 kb where modification of primers to contain comparable restriction sites has prevented efficient amplification (E.K. Green et al., in prep.).

Directional cloning using restriction enzymes also often requires long, and/or separate, sequential digestions (each requiring PCR product purification and tailoring of buffer composition to the particular restriction enzymes used) to maximize the yield of cohesive ends produced and minimize the degree of star activity generated. The rapid hydrolysis and relatively wide temperature and buffer activity range of UDG makes it possible to avoid these steps. UDG may simply be added to the reaction products immediately following thermal cycling, and incubated for 30 min, followed by alkali treatment, purification, and ligation. Alkali treatment, which cleaves at abasic sites, following incubation with UDG, substantially increases the subsequent availability of cohesive ends for ligation, thus affecting cloning efficiency, consistent with previous studies on the specificities and kinetics of dU excision by UDG.^(18,19)

Potential advantage can further accrue from how the mimics behave following ligation. DNA segments joined together via ligation of restriction enzyme-generated cohesive ends retain the capacity to be recut by that enzyme at the original junction, unless subsequently modified, for example, by methylation. Our data show that using UDG mimics, it is possible to balance cloning efficiency and accuracy with the ability to recreate or abolish the original restriction site following successful ligation (see Tables 1 and 2). This facet of the UDG-based strategy may have particular value in constructing large or chimeric genes using PCR subfragments where loss of the recognition sequence following ligation is an advantage.

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