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scraped into 1 ml of PBS without Mg^{2+} or Ca^{2+}, centrifuged at 5,000 *g* for 4 min, resuspended in 100 µl of lysis buffer (102 mM $Na_2HPO_4 \cdot 7H_2O$, 102 mM KH_2PO_4, pH 7.4) and lysed by four freeze-thawing cycles, centrifuged at 12,000 *g* for 10 min at 4°C, and the supernatant retained for activity assays. Reactions (50 µl) contained 0.625 mM $MgCl_2$, 13.9 mM NaCl, 0.1 mM EDTA, 7 mM Tris-HCl (pH 7.4), 1 mM phosphoribosyl pyrophosphate (PRPP), and 13 µM [8-^{3}H]hypoxanthine (2.3 Ci/mmol). At various times 10 µl was removed and added to 5 µl 100 mM EDTA on ice to halt the reaction and spotted onto a Whatman DE-81 filter disk. The disks were dried, washed twice in 10 mM ammonium formate (10 ml/disk) to precipitate the [^{3}H]IMP, and twice in absolute ethanol (10 ml/disk). The disks were dried, and tritium incorporation was measured by liquid scintillation. Enzymatic activity of HPRT was measured by fluorography in polyacrylamide gels as described[25].

Fluorescent in situ hybridization. *HPRT* PAC71G04 and pBAC108L (ref. 4) were labeled with biotin using BioNick labeling system (Gibco BRL) and digoxigenin using Dig-Nick Translation Mix (Roche), respectively. After hybridization and washing, the glass slide was incubated with blocking buffer (PBST containing 1% BSA, 0.2% fish gelatin, and 25 mM glycine; pH 8) at room temperature for 30 min, and with 2 µg/ml Cy3-conjugated mouse anti-dig antibody (Jackson ImmunoResearch, West Grove, PA) and 10 µg/ml Alexa 488-conjugated NeutrAvidin (Molecular Probes, Eugene, OR) for 1 h and then washed 4× with PBST over 1 h. Nucleic acids were stained with 50 nM TOTO-3 iodide (Molecular Probes) in PBS at room temperature for 5 min. After final washing with PBS for 5 min, the sample was mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images were obtained using a Bio-Rad MRC1000 confocal laser scanning microscope (Bio-Rad, Hercules, CA) equipped with an Argon/Krypton laser and coupled to a Nikon Diaphot 200 inverted microscope.

Acknowledgments

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DNA cloning by homologous recombination in *Escherichia coli*

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The cloning of foreign DNA in *Escherichia coli* episomes is a cornerstone of molecular biology. The pioneering work in the early 1970s, using DNA ligases to paste DNA into episomal vectors, is still the most widely used approach. Here we describe a different principle, using ET recombination[1,2], for directed cloning and subcloning, which offers a variety of advantages. Most prominently, a chosen DNA region can be cloned from a complex mixture without prior isolation. Hence cloning by ET recombination resembles PCR in that both involve the amplification of a DNA region between two chosen points. We apply the strategy to subclone chosen DNA regions from several target molecules resident in *E. coli* hosts, and to clone chosen DNA regions from genomic DNA preparations. Here we analyze basic aspects of the approach and present several examples that illustrate its simplicity, flexibility, and remarkable efficiency.

Recently we described a new way to use homologous recombination for DNA engineering in *E. coli*. We termed the approach "ET recombination" because we first uncovered it using the Rac phage protein pair, RecE/RecT, and then later showed that the equivalent lambda phage protein pair, Redα/Redβ, also worked[1,2]. Our original goal was to develop a simple method to engineer bacterial artificial chromosomes (BACs)[1–4]. However, the approach worked so well that other applications became apparent, such as rapid creation of new *E. coli* strains through direct targeting of the *E. coli* chromosome[1], rapid generation of gene targeting constructs for use in mouse embryonic stem (ES) cells[5], and a new way to accomplish site-directed mutagenesis in plasmids[1] and BACs[6,7]. Recently three other groups, aiming to develop a better way to directly target the *E. coli* chromosome, have also demonstrated that ET recombination initiat-

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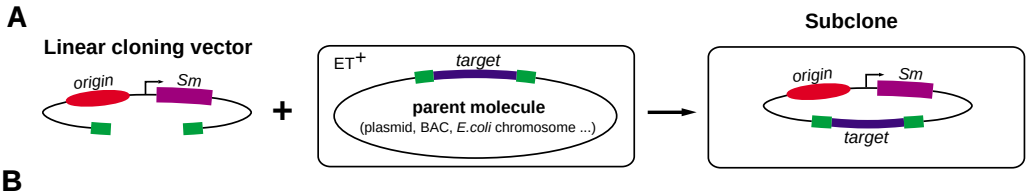
ed by the Redα/Redβ pair works with striking efficiency[8–11].

To date, all applications of ET recombination, whether with RecE/RecT or Redα/Redβ, have modified pre-existing replication-competent molecules[1–11]. Here we show that ET recombination can be applied to clone and subclone DNA regions from a DNA source into a plasmid. This new application provokes very different practical implications.

As described elsewhere, ET recombination works well with homology regions that are short enough to be included in synthetic oligonucleotides[1,10–12]. In a convenient application, these oligonucleotides also contain a primer site for PCR amplification of a selectable gene, such as an antibiotic resistance gene. Hence the PCR product contains the selectable gene flanked by two homology arms. In the presence of either RecE/RecT or Redα/Redβ, and the absence of RecBCD, homologous recombination between the homology arms and the chosen target regions integrates the selectable gene. In other words, the homology arms define the integration site, and thereby an existing replication-competent molecule is modified[1]. Here, we alter this basic strategy so that the PCR product is a plasmid backbone, including origin of replication and selectable gene, flanked by homology arms. In this application, the homology arms define the region that is to be copied into the plasmid.



B

Example	Linear cloning vector	ET expression	*E. coli* host	Target	Parent molecule	Total colonies	Percentage correct (out of 18)
1	p15A+*Cm*	Endogenous RecE/RecT	JC9604	2.3 kb, endogenous *lacZ* gene	*E. coli* chromosome	290	100
2	p15A+*Cm*	pBAD-αβγ	HB101	2.3 kb, endogenous *lacZ* gene	*E. coli* chromosome	370	94
3	p15A+*Km*	pBAD-recET	JC5519	0.8 kb, gentamicin gene	pFastBac1 (high-copy plasmid)	>3,000	100
4	p15A+*Km*	pBAD-αβγ	HS996	28 kb, intron3 of *mAf4*	BAC	160	83
5	p15A+*Amp*	pR6K116/BAD /αβγ	HS996	19kb, exons 2-3 of *mAf4*	BAC	>2,000	83

Figure 1. Subcloning by ET recombination. (A). Diagram of the strategy showing the linear cloning vector carrying an *E. coli* plasmid origin and an antibiotic selectable marker (*Sm*) gene flanked by two oligonucleotide homology arms (green blocks). The linear cloning vectors were PCR amplified using oligonucleotides containing the homology arms at their 5′ end, and PCR primers at their 3′ ends for amplification of the plasmid origin/antibiotic resistance gene cassettes. (B). Summary of five examples of the reaction diagrammed in (A). The linear cloning vector column states the plasmid origin/selectable marker cassette joined, by PCR, to the homology arms used. The *p15A+Cm* (chloramphenicol resistance gene) cassette was PCR amplified from pACYC184. The *p15A+Km* (kanamycin resistance gene) was PCR amplified from pACYC177. The *p15A+Amp* (ampicillin resistance gene) was PCR amplified from pACYC177. The ET expression column states the source of expression of the ET genes. Constitutive expression of RecE/RecT in JC9604 was used in example 1. pBADαβγ (ref. 2) and pBAD-recET (refs 1,12) have been published. pR6K116/BAD/αβγ is described here. The *E. coli* host column states the strain used. The target column states the DNA region that was subcloned into the linear cloning vectors. The parent molecule column states the source of the target region present in the ET+ *E. coli* host. The total colonies column presents the number of colonies that grew after selection for the antibiotic resistance gene included in the linear cloning vector. The percentage correct column presents results from restriction enzyme, and in certain cases, DNA sequence, analysis of 18 colonies selected from each experiment. All incorrect colonies observed in examples 2, 4, and 5 were empty circularizations of the linear cloning vector. The lengths of homology regions used, in nucleotides, were as follow: examples 1 and 2, 59 (left arm) and 56 (right arm); example 3, 47 and 51; example 4, 64 and 61; example 5, 55 and 52.

To test whether ET recombination could be used in this way, we first tried subcloning various regions from replication-competent molecules present in *E. coli*. The experimental strategy is illustrated in Figure 1A. Several variations and targets were tested by choosing different homology arms (by oligonucleotide synthesis) to flank various target regions, including the endogenous *lacZ* gene on the *E. coli* chromosome (Fig. 1B, examples 1 & 2), a part of a high-copy plasmid (example 3), and parts of a BAC (examples 4 and 5). The PCR products included the p15A plasmid origin combined with different selectable genes. They were electroporated into ET-competent *E. coli* hosts that carried the respective targets. Antibiotic-resistant colonies were examined for the intended recombination event. These exercises were remarkably efficient (Fig. 1). In all cases, the intended target region defined by the flanking homology arms was fully inserted into the episome without any detectable mutational errors (data not shown). In particular, the subcloning of *intron 3* of the mouse *Af-4* gene[13] from a BAC (Fig. 1B, example 4) is notable. At the time we designed the experiment, we did not know the size of this target region but only knew the sequences of the flanking exons. From the ET subcloning product and subsequent characterization of the mouse *Af-4* gene, we determined that the target region was 28 kb. All clones examined faithfully copied this 28 kb piece into the subclone (data not shown).

In examples 1 and 4 of Figure 1, we examined the target region in the parent molecule after ET recombination to determine whether it had been copied into the subclone, or excised from the parent. In both cases, the target region remained in the parent, thus it had been copied into the subclone (data not shown).

We achieved good efficiencies in these subcloning experiments, but were surprised because we had anticipated that the major competing product, derived from empty circularization of the linear vector, would be dominant. Empty circularization products were observed. They accounted for all of the incorrect resistant colonies examined (Fig. 1). Thus empty circularization is the most important source of background. However, the intended ET recombinants were clearly the most abundant products.

These subcloning exercises showed that ET recombination is an efficient way to amplify a chosen target region from the complex background presented by the *E. coli* genome. This subcloning advance resembles work using recombinogenic targeting in yeast, which is a conceptually similar approach using the implicit recombination potential of yeast[14–17]. Recombinogenic targeting and a related, more restricted approach (transformation-associated recombination), have also been applied to clone directly from samples of genomic DNA (ref. 18). Hence, we next applied ET recombination to the more diffi-

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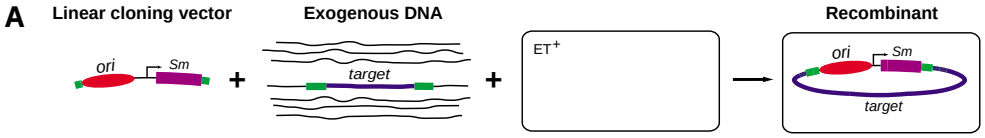


B

Example	Linear cloning vector	Target	Exogenous DNA source	ET expression	Total colonies	Percentage correct (out of 18)
1	*ColE1+Km*	4.5 kb, *lacI+lacZ* gene	*E. coli* chromosome DNA	YZ2000	174	100
2	*p15A+Km*	1.1 kb, *bla* gene in chromosome	Yeast genomic DNA	YZ2000 +pBAD/recT	58	33
3	*ColE1+Cm*	1.0 kb, *neo* gene in chromosome	Mouse ES cell genomic DNA	YZ2000+ pR6K116/ BAD/recT	32	17

Figure 2. Cloning by ET recombination. (A) Diagram of the strategy with notations as in Figure 1A, except that the target DNA was included in total, genomic DNA purified from *E. coli*, yeast, or mouse embryonic stem cells. (B) Summary of three examples of the reaction shown in (A). The linear cloning vectors were made by PCR from pZeRO-2 (example 1), pACYC177 (example 2), and a derivative of pZeRO-2 that includes the chloramphenicol resistance gene (example 3). In example 1, a part of the *lac* operon was targeted. In examples 2 and 3, antibiotic resistance genes previously integrated into the host genome and determined to be present as single copies (data not shown) were targeted. They were not selected for in the ET cloning step but facilitated the subsequent analysis of colonies that grew up after selection for the antibiotic gene in the linear cloning vector. The lengths of homology regions used were as follow: example 1, 49 and 50; example 2, 61 and 61; example 3, 74 and 76. See Figure 1B for other details.

cult task of direct cloning from complex mixtures of exogenous DNA.

Figure 2 summarizes results from three experiments where target regions from total genomic DNAs of *E. coli*, yeast, and mouse were cloned. As expected from the differing complexities of these genomes, fewer correct clones were identified from mouse genomic DNA than from yeast or *E. coli* DNA. This probably reflects the physical limitation imposed by co-electroporation of the linear cloning vector with total genomic DNA. With more complex genomes, fewer cells will be co-electroporated with both the targeting vector and the target. These experiments demonstrate directed cloning of chosen DNA regions from exogenous samples by ET recombination. Of the three examples shown, target cloning from mouse genomic DNA was the most challenging. The result presented relied on the following four refinements.

First we analyzed the empty circularization reaction so that this source of background could be reduced. The presence or absence of 5′ phosphates on the linear vector ends made no difference (data not shown). We then looked at the effect of including short repeats in the oligonucleotides (Fig. 3). When repeats of six bases or more were present, either internally or directly at the 5′ ends, empty circularization of the linear vector was promoted (Fig. 3C, x-c, x-d, x-e, and data not shown). Therefore, we recommend that the sequences chosen for use as homology arms should not include repeats longer than 5 bp. Similarly, repeats longer than 5 bp elsewhere in the linear vector may also be deleterious and should be avoided, particularly in the regions flanking the origin/selectable marker cassette.

Second, we modified JC8679, the original *sbcA* (RecE/RecT$^+$) strain described by Clark[19]. Although JC8679 can be used for DNA engineering exercises in certain cases, it retains all *E. coli* restriction/modification systems. Consequently it is not an optimal cloning host. The implicit ET recombination potential of JC8679 was used to delete its restriction/modification systems and the endogenous lac operon, to create YZ2000 (*sbcA*, *recBC*, *lacI-Z*, *mcrA*, *mcrBC*, *mrr*, *hsdMRS*; data not shown).

Third, we made pR6K116, a high-copy plasmid containing the R6K origin of replication and the *pir-116* gene from the *E. coli* strain BW23474 (ref. 20). pR6K116 was stable under selection, is replicated at a copy number equivalent to pUC *ColE1* plasmids, and is compatible with *ColE1* and p15A replication origins (data not shown). By placing the genes for ET recombination (RecE/RecT, Redα/Redβ, and Redγ) into pR6K116 (Fig. 3D), we were able to use a *ColE1* plasmid origin in the linear cloning vector. This year, three other ways to express Redα/Redβ/Redγ have been published[9–11]. These different configura-

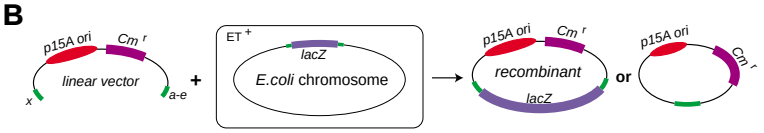
A

left: x, 5′ GCCAGCTGGCGCAGGTAGCAGAGCGGGTAAACTGGCTCGGATTAGGGCCGCAAGATTACGCCCCGCCCTGCCAC 3′

right: a, 5′ CGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTCACAACTTATATCGTATGGGG 3′
b, 5′ CGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTCTTACAACTTATATCGTATGGGG 3′
c, 5′ CGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTCTTGCACAACTTATATCGTATGGGG 3′
d, 5′ CGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTCTTGCGGACAACTTATATCGTATGGGG 3′
e, 5′ CGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTCTTGCGGCCACAACTTATATCGTATGGGG 3′

homology arm — repeat — PCR primer

B



C

Combination of primers	Colonies	Percentage correct ET recombinants	Percentage empty circularizations
x-a	525	100	0
x-b	514	100	0
x-c	793	70	30
x-d	2,160	23	77
x-e	5,640	6.0	94

D

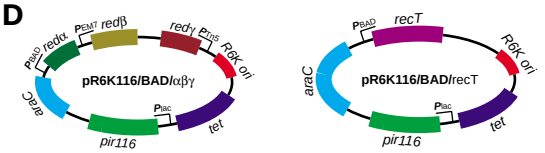


Figure 3. Additional aspects involved in ET subcloning. (A-C) Effect of short repeats in the promotion of empty circularizations. (A) Sequences of the oligonucleotides used to create a series of linear cloning vectors by PCR of pACYC184. The left oligonucleotide, x, was common to all in this series. The right oligonucleotides, a-e, included additional sequences (shown in bold) that were repeats of the underlined sequences in x. The sequences corresponding to the PCR primer regions, homology arms, and repeats are indicated. (B) Diagram of the strategy used to analyze the effect of short repeats on the efficiency of subcloning a part of the lacZ gene resident on the host E. coli chromosome. Results shown are from experiments using JC8679 (ref. 19). Aspects of the same experiment were repeated using different sources of ET gene expression with, qualitatively, the same results (data not shown). (C) Summary of results. In all cases, the 5¢ termini of the oligonucleotides were not phosphorylated. (D) Diagrams of the plasmids, pR6K116/BAD/abg and pR6K116/BAD/recT.

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tions should also be useful for ET cloning, and it would be interesting to compare all the available approaches for efficiencies and conveniences.

Fourth, during fundamental studies on the mechanism of double-stranded break repair employed by RecE/RecT and Redα/Redβ (ref. 12), we observed that ET recombination efficiencies improve significantly when the annealing protein, RecT or Redβ, is overexpressed with respect to its partner exonuclease, RecE or Redα. To overexpress RecT in YZ2000, it was cloned into pR6K116 to create pR6K116/BAD/recT (Fig. 3D).

Implementing aspects of the four lines of work described above, a chosen target region in mouse genomic DNA was amplified to be present in one of every six colonies on selection plates (Fig. 2B, example 3). All other colonies examined were empty circularizations. Further exploration of the variables involved are likely to improve on this efficient outcome.

Here we show that ET recombination can be applied to two fundamental tasks in molecular biology. First, chosen DNA regions can be subcloned by a new approach that is simple, efficient, and more flexible than the existing methodology. Second, as with PCR, DNA regions can be amplified from mixtures. Unlike PCR, the regions are amplified in *E. coli*, not in vitro, and thus are subject to review by the *E. coli* replication machinery. Both of these advances present new options for DNA engineering.

Experimental protocol

All methods were essentially the same as described[1,2,12,21]. In our standard format for subcloning by ET recombination (Figs 1 and 3), a 50 μl aliquot of ET-competent (ET^+) *E. coli* cells harboring the parent molecule was electroporated with 0.3 μg of a linear cloning vector. After electroporation, colonies that grew under selection for the antibiotic resistance gene were examined for the intended ET subcloning product. For cloning by ET recombination (Fig. 2), 5 μg of genomic DNA was mixed with 0.5 μg of the appropriate linear cloning vector and co-electroporated into a 50 μl aliquot of ET^+ cells. To improve the solubility of the total genomic DNA preparations, they were either precleaved using rare cutting restriction enzymes or sheared through a 0.22 μm needle. Recombinants were identified by selection for the antibiotic gene present on the linear cloning vector and subsequent DNA analysis. Oligonucleotide sequences were as shown:

Figure 1B (examples 1 and 2)

Left:5′-TGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCAGCACGACGTTGTAAAACGACTCGAGACAACTTATATCG-TATGGGGC3′

Right: 5′-TGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGT-CAAAAATAATAATAACCGGGGTACCTTACGCCCCGCCCTGCCACTC-3′

Template: pACYC184

Figure 1B (example 3)

Left: 5′-TGATATCGACCCAAGTACCGCCACCTACAATTCGTTCAAGC-CGAGGATCCTTAATAAGATGATCTTCTTGAG-3′

Right: TACAGTTTACGAACCGAACAGGCTTATGCAACTGGGTTCGT-GCCTTCAGAATTCTGATTAGAAAAACTCATCGAGC-3′

Template: pACYC177

Figure 1B (example 4)

Left: 5′-TGTAGCTGAGCCCAGGGGCAAGGCTGCTTTGTACCAGCCT-GCTGTCTCGGGGGCATCACCTGGAATTCTTAATAAGAT-GATCTTCTTGAG-3′

Right:5′-TGGGGTGTCAACCTCAGGCTTTCTCACACG-CAAACAGGTAGGGACTTGCACCCCTACACACCGAATTCTTA-GAAAAACTCATCGAGCATC-3′

Template: pACYC177

Figure 1B (example 5)

Left: 5′-TGCAAAAGGCGATGAGCTATCAAGTCGGATCCAGACCAT-GCTGGGTGACTATGAGGAGAGGAATTCGCGCTAGCGGAGTGTAT-ACTGGC-3′

Right:5′-TCCGCTGCCCCTTGATACATGGGGTGGACAGGATTAAG-GAAGGCCTGGGGTGTCAACCTCAGCTCGAGTGAAGACGAAAGGGC-CTCGTG-3′

Template: pACYC177

Figure 2B (example 1)

Left: 5′-TCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGT-GTCAACGTATACTTCCGCTTCCTCGCTCACTGAC-3′

Right:5′-TCCCGATTTGGCTACATGACATCAACCATATCAGCAAAAGT-GATACGGGTATACTCAGAAGAACTCGTCAAGAAGG-3′

Template: pZErO-2

Figure 2B (example 2)

Left:5′-TATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT-CACTGATTAAGCATTGGTAATTAATAAGATGATCTTCTTGAG-3′

Right: 5′-CATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAAC-CATTATTATCATGACATTAACCTATTAGAAAAACTCATCGAGCATC-3′

Template: pACYC177

Figure 2B (example 3)

Left: 5′-TGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGC-CTTCTATCGCCTTCTTGACGAGTTCTTCTGATTCCGCTTCCTCGCT-CACTGAC-3′

Right: 5′-TTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCC-CCAGCTGGCAATTCCGGTTCGCTTGCTGTCCATTACGCCCCGCCCT-GCCACTCATC-3′

Template: pYZCC (a derivative of pZErO-2 but chloramphenicol resistant)

Further information can be found at http://www.embl-heidelberg.de/-ExternalInfo/stewart/index.html.

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