RECOMBINEERING: A POWERFUL NEW TOOL FOR MOUSE FUNCTIONAL GENOMICS

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Highly efficient phage-based *Escherichia coli* homologous recombination systems have recently been developed that enable genomic DNA in bacterial artificial chromosomes to be modified and subcloned, without the need for restriction enzymes or DNA ligases. This new form of chromosome engineering, termed recombinogenic engineering or recombineering, is efficient and greatly decreases the time it takes to create transgenic mouse models by traditional means. Recombineering also facilitates many kinds of genomic experiment that have otherwise been difficult to carry out, and should enhance functional genomic studies by providing better mouse models and a more refined genetic analysis of the mouse genome.

MOUSE GENOMIC TECHNOLOGIES

PHAGE-BASED RECOMBINATION Bacteria, such as *Escherichia coli*, encode their own homologous recombination systems. Viruses or phages that inhabit bacteria also often carry their own recombination functions, which can work with, or independently of, the bacterial recombination functions.

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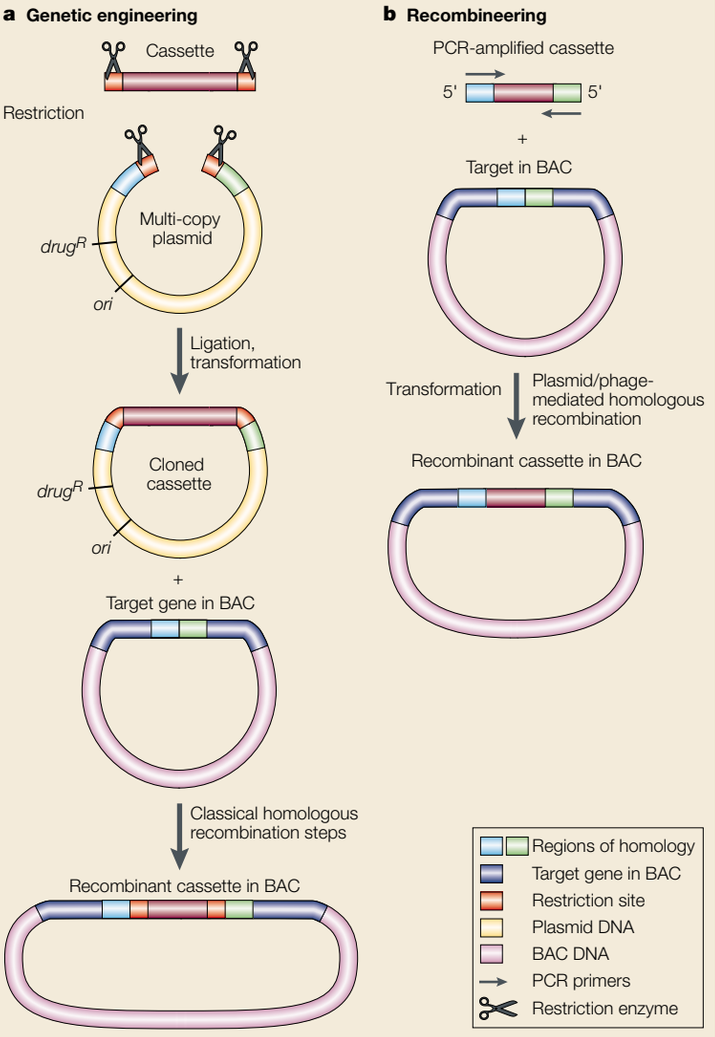
During the next few years, the sequence of the human genome will be completed and annotated. The next challenge will be to determine how each of these genes functions alone and with other genes in the genome, to understand the developmental programme of a human. Given that there are many genes that need to be characterized and the fact that a lot of them will not be related to genes with known function, this will be a daunting task. Much of our understanding of these genes will therefore have to come from studies of model organisms.

The mouse is an ideal model organism for these types of study. Not only are the mouse and human genomes very similar, but also transgenic and knockout technologies, developed during the past two decades, allow gene function studies in the mouse that are not possible in most other organisms. Null mutations, as well as subtle missense or gain-of-function mutations, can be introduced into virtually any gene in the mouse germ line using homologous-recombination-based, gene-targeting technology. In addition, the conditional inactivation of gene expression *in vivo* can be used to inactivate a gene in only a subset of cells or at well-defined stages of development[1–4] (for more on this topic, see the accompanying review by Mark Lewandoski on p743 of this issue).

A limitation of most of these technologies is that they require the production of complicated targeting and selection constructs. The ability to generate these constructs is often limited by the availability of appropriate and unique restriction-enzyme cleavage sites in both cloning vectors and genomic DNA (BOX 1). Other types of functional genomics experiment also require the manipulation of large segments of DNA, which is difficult to do using standard recombinant DNA techniques, because many cloning vectors do not have sufficient capacity or do not tend to tolerate large inserts. The development of PHAGE-BASED HOMOLOGOUS RECOMBINATION systems in the past three years[5–8] has greatly simplified the generation of transgenic and knockout constructs, making it possible to engineer large segments of genomic DNA, such as those carried on BACTERIAL ARTIFICIAL CHROMOSOMES (BACs) or P1 artificial chromosomes (PACs), that replicate at low-copy number in *Escherichia coli*. Using phage recombination to carry out genetic engineering has been called recombinogenic engineering[9] or recombineering. Recombineering offers exciting new opportunities for creating mouse models of human disease and for gene therapy. Here, we contrast and compare these new phage systems with

Box 1 | **Classical recombinant DNA technology versus recombineering**

Traditional genetic engineering technology, which uses restriction enzymes and DNA ligase to cut and rejoin DNA fragments (see figure part **a**), breaks down when cloning vehicles and the target site contain hundreds of kilobases of DNA. This is because even rare restriction-enzyme sites occur frequently on large DNA molecules, such as bacterial artificial chromosomes (BACs) and the bacterial chromosome, making the finding of unique sites almost impossible. Furthermore, the *in vitro* manipulation of large linear DNAs of this length is extremely difficult; once BAC cloning technology became available in *Escherichia coli*, modifying the BAC clones became the primary problem. Problems associated with the traditional approach have been overcome by developing recombineering technology, which uses homologous recombination functions that are encoded by phages (see figure part **b**). The crucial advance in the recombineering methodology is the use of phage recombination functions that generate recombinant molecules using homologies of 50 bp (or less); homologous regions for recombination usually need to be ~500 bp in length, whereas in recombineering approaches, they need be only 40–50 bp in length. (*ori*, origin of replication.)



Genetic engineering steps to generate a BAC recombinant include:

- Cleaving a cassette DNA with a restriction enzyme.
- Cleaving a target DNA on a plasmid with a restriction enzyme.
- Ligating the cassette to the plasmid.
- Transforming the plasmid into competent bacterial cells.
- Selecting drug-resistant (*drug*^{R}) clones.
- Isolating the transformant and verifying the cloned cassette.
- Transforming the cloned cassette into a BAC-containing bacterial strain to introduce the cloned cassette into the BAC by homologous recombination.

Recombineering steps to generate a BAC recombinant include:

- Amplifying a cassette by PCR with flanking regions of homology.
- Introducing phage recombination functions into a BAC-containing bacterial strain, or introducing a BAC into a strain that carries recombination functions.
- Transforming the cassette into cells that contain a BAC and recombination functions.
- Generating a recombinant *in vivo*.
- Detecting a recombinant by selection, counterselection or by direct screening (colony hybridization).

earlier, more classical bacterial recombination systems that have been used for engineering genomic DNA on BACs or PACs.

Chromosome engineering in yeast

E. coli has traditionally been the main organism in which to carry out genetic engineering for genetic studies of bacteria, and also for constructing recombinant DNA molecules for use in other organisms. Most *E. coli* cloning methods use restriction enzymes to cleave DNA, and DNA ligases to join DNA fragments. These cleavage and joining reactions are the basis for creating recombinant molecules, and for cloning DNA into phage and PLASMID vectors for additional modification and amplification. In *Saccharomyces cerevisiae*, the DNA double-stranded-break and -repair recombination pathway is very efficient[10], making yeast a useful organism for creating recombinant DNA molecules by homologous recombination. These recombination pathways recombine transformed, linear, double-stranded DNA (dsDNA) with homologous sites in the yeast genome. Moreover, recombination occurs proficiently with even short stretches of homologous sequence, thereby allowing recombinant DNA to be generated *in vivo* without the use of restriction enzymes and DNA ligases[11–13].

Homologous recombination has proved to be extremely useful for analysing the function of genes in the yeast genome. In 1993, Agnès Baudin and colleagues[13] showed that it is possible to delete a yeast gene and replace it with a yeast selectable marker, such as *HIS3*, using homologous recombination. In these

BACTERIAL ARTIFICIAL CHROMOSOME (BAC). A cloning vector derived from a single-copy F-plasmid of *Escherichia coli*. It carries the F replication and partitioning systems that ensure low-copy number and faithful segregation of plasmid DNA to daughter cells. Large genomic fragments can be cloned into F-type plasmids, making them useful for constructing genomic libraries.

Box 2 | YACs versus BACs and PACs for recombineering

Yeast artificial chromosomes (YACs) were developed for cloning large fragments of genomic DNA into yeast, whereas bacterial artificial chromosomes (BACs) and P1 artificial chromosomes (PACs) were developed for cloning large genomic fragments into *Escherichia coli*. Each vector type has its own inherent advantages and disadvantages. Those with regard to YACs versus BACs or PACs are listed below.

Advantages

- **YACs can contain megabase-sized genomic inserts, whereas BACs and PACs carry inserts of ~200–300 kb or less[70,71].**

Disadvantages

- **YAC DNA is difficult to purify intact and at a sufficiently high concentration for generating YAC transgenic mice. BAC and PAC DNA is easy to purify intact[72,73], and BAC and PAC transgenic mice can be produced in large numbers[41,74,75].**
- **A single yeast cell might contain both wild-type and modified YACs[76]. A bacterial cell usually carries a single BAC.**
- **YACs are often chimeric; a single YAC can contain DNA from multiple locations in the genome. BACs and PACs are rarely chimeric.**
- **Yeast recombination is very potent and always active, and so can generate deletions and other rearrangements in a YAC. *E. coli* recombination is prevented in RECA mutants and is turned on when needed, so reducing unwanted rearrangements in BACs or PACs.**
- **Manipulating recombinant YACs that are generated in yeast is laborious and usually requires YACs to be transferred to *E. coli* for subsequent manipulation. BAC and PAC modification occurs directly in *E. coli*, eliminating the need for DNA transfer.**

experiments, *HIS3* was amplified with chimeric PCR primers that contained 19 bases of *HIS3* homology at their 3′ ends and 35–51 bases at the 5′ ends containing homology to the yeast gene to be deleted. The yeast gene was then replaced with *HIS3* by introducing the amplified *HIS3*-targeting cassette into yeast and selecting for *HIS3* recombinants. Because oligonucleotide synthesis and PCR amplification are the only molecular steps in this approach, it can be used to rapidly generate large numbers of gene deletions. In fact, most genes in the yeast genome have now been deleted using this simple technique[14,15]. In addition to yeast knockouts, yeast homologous recombination has facilitated the generation of targeting vectors for mouse knockouts[16–18], and has been used to subclone DNA by GAP REPAIR into yeast SHUTTLE VECTORS for functional analysis[19,20].

Although yeast homologous recombination has allowed recombinant DNA to be generated *in vivo* with the minimal use of restriction enzymes and DNA ligase, it has several inherent limitations (BOX 2). Owing to these limitations, and to the advantages of using the more stable bacterial BAC and PAC clones, a tightly regulated homologous recombination system in bacteria has been developed for modifying DNA that is cloned into BAC and PAC vectors.

Early strategies for DNA engineering in *E. coli*

Unlike in yeast, linear dsDNA is unstable in *E. coli* due to the presence of the ATP-dependent, linear-dsDNA exonuclease, RecBCD[21–26]. However, *E. coli* strains that lack RecBCD by virtue of a *recBC* mutation can be transformed by linear dsDNA, provided that they also have *sbcB* and *sbcC* mutations, which restore recombination activity to *recBC* mutants[24,26–28]. These exonuclease-deficient strains provided one of the first *in vivo* cloning systems for *E. coli* and have been used for several applications, including recombining linear dsDNA with circular chromosomal DNA[29], introducing drug-selectable markers into the *E. coli* chromosome, and for mutagenizing various plasmid-cloned genes[30].

RecBCD-exonuclease-deficient strains have also been used to subclone PCR products into plasmids by a process called *in vivo* cloning[31,32]. *In vivo* cloning (FIG. 1) is similar to yeast gap repair; linear PCR products with terminal sequences that match those at the two ends of a linearized plasmid vector are co-transfected with vector DNA into *E. coli recBC*$^{-}$ *sbcBC*$^{-}$ cells (FIG. 1). Recombination between the two sets of homologies generates a circular plasmid by gap repair that can replicate and be selected in *E. coli*. This cloning method removes the need for enzymatic treatment of the PCR product or for *in vitro* ligation. Unfortunately, in all of these examples, recombination is restricted to special RecBCD-deficient strains and is therefore not universally applicable. A more serious shortcoming of these strains is that the recombination pathway is constitutively active in them, causing rearrangements and deletions between the repeat sequences that are found in most BAC and PAC clones.

***Chi*-stimulated recombination.** Chi-stimulated recombination provides a way to modify genomes with linear dsDNA in wild-type cells. Chi sites (5′ GCTGGTGG 3′), when incorporated at each end of linear dsDNA, protect the DNA from digestion by RecBCD and provide activation sites on the DNA for RecBCD-mediated recombination in wild-type cells[33,34]. On encountering a Chi site, the exonuclease activity of RecBCD is attenuated, but because it maintains its helicase activity, it acts on the linear dsDNA to produce a long, single-stranded DNA (ssDNA) tail with a 3′ end. This ssDNA tail is postulated to be the substrate for DNA strand transfer that is promoted by RecA (see link to the RecA web site) and single-stranded binding (SSB) proteins[35,36]. Recombination can then occur between the linear dsDNA fragment with its single-stranded ends and homologous sequences on the *E. coli* or artificial chromosome. Chi sites can flank several kilobases of heterologous sequence and still work[37].

Using Chi-stimulated homologous recombination, the first coding exon of the zebrafish *gata2* gene on a BAC has been replaced with a reporter gene that encodes green fluorescent protein (GFP)[38]. Zebrafish embryos that were microinjected with the modified BAC were less mosaic and had improved GFP expression in haematopoietic progenitor cells compared with embryos that were injected with smaller clones on multi-copy plasmid constructs. The pattern of GFP expression also closely resembled that of the endogenous *gata2* gene. Although similar experiments have not been done with mouse DNA, Chi-stimulated recombination should work with the cloned DNA of any species. However, a principal limitation of Chi-stimulated homologous recombina-

PLASMID
An autonomously replicating DNA that is often marked with a gene that encodes drug resistance, which allows selection for cells that carry the plasmid.

HIS3
A yeast selectable marker that encodes an enzyme required for histidine (His) biosynthesis. *HIS3* yeast mutants cannot grow in media without His. On *HIS*-deficient medium, recombinants that restore the wild-type gene are able to grow again.

GAP REPAIR
A linear plasmid vector (gapped vector) can be circularized by homologous recombination between its ends and a target DNA.

SHUTTLE VECTOR
A plasmid that can be moved from one species to another, such as plasmids that contain origins of replication for both yeast and bacterial hosts.

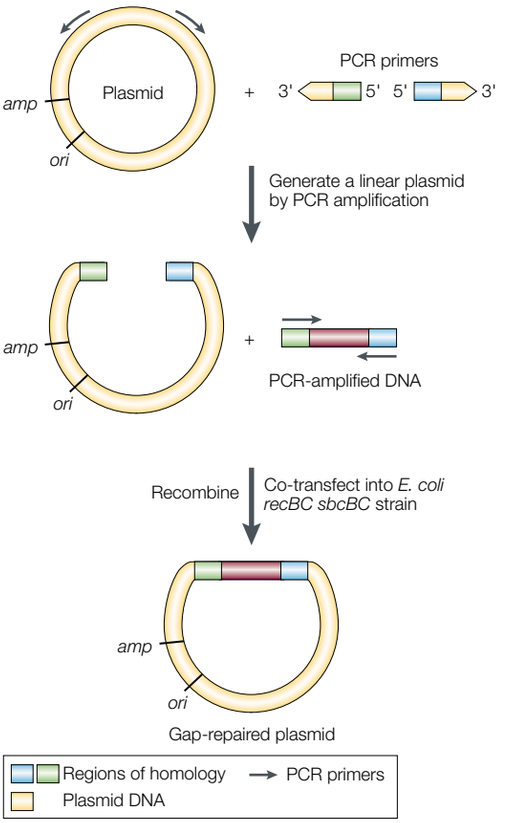


Figure 1 | ***In vivo* cloning.** This method of *in vivo* cloning uses two linear DNAs — a vector and a target DNA — which carry stretches of homology to each other at their ends. Both linear DNAs are electroporated into competent cells to allow homologous recombination between them, thereby repairing the plasmid DNA by closing the circle. (*amp*, ampicillin resistance gene; *ori*, origin of replication.)

tion is that it is very inefficient, even with long stretches of homology, which requires that POSITIVE–NEGATIVE SELECTION is used to find the rare recombinants.

Providing RecA function. RecBCD does not degrade circular DNA. Therefore, genomes can be modified in wild-type *E. coli* strains using circular dsDNA targeting cassettes, provided that they are also wild-type for *recA* (*recA*$^+$)[39–41], as RecA function is essential for integrating circular DNA by homologous recombination. Because the *E. coli* host that is used to generate most BAC libraries (DH10B) is defective for *recA*, modifying BAC DNA in this host is done through a special vector that carries the wild-type *recA* gene[41] (FIG. 2). When *E. coli* is transformed with this vector, it becomes competent to carry out homologous recombination. The vector has three other important properties: it contains a tetracycline resistance (Tet^R) cassette that allows it to be selected with tetracycline; it has a temperature-sensitive origin of replication (see below); and it is designed to carry cloned segments that are homologous to targets in either the bacterial chromosome or on BAC genomic clones.

Using this vector, a *lacZ* gene was inserted into the mouse *Ru49* zinc-finger gene carried on a 131-kb BAC[41]. In these experiments, the *lacZ* gene was flanked by 500-bp regions of homology to the *Ru49* gene and cloned into the special vector (FIG. 2). It was then transformed into *E. coli* cells that carry the BAC at 42 °C. Recombination between the vector and the *Ru49* gene can then occur through one of the two 500-bp homologies to insert the entire vector into the BAC, allowing its selection as a Tet^R colony. On regrowth of this colony at 37 °C, a fraction of the integrants will undergo a second recombination event (resolution) through either homology arm. Depending on which homology arm is used, the resolved BAC can either regenerate the original BAC or create the intended modification by leaving the *lacZ* fusion in the *Ru49* gene on the BAC. Transgenic mice that carry this modified BAC showed *lacZ* expression in the brain[40]. Although the first test of this recombination system was done with BACs, it could be adapted to modify PACs, P1 and other vectors that are propagated in recombination-deficient *E. coli*.

There are two important limitations of this system. First, the vector must be appropriately pre-engineered to contain large DNA segments that are homologous to the target in the BAC. Second, the special vector that carries the *recA* gene is present in the cells for several generations of growth to carry out the two recombination steps described. This is a substantial amount of growth time during which other unwanted homologous recombination events can occur between DNA repeats in genomic BAC clones[42]. Rearrangements caused by these unwanted recombinations often create a growth advantage over the original BAC strain. So, BACs from such cultures might be correctly engineered at the target, but might have rearrangements elsewhere.

Phage-encoded recombination systems

Rac-encoded RecET system. In 1998, Francis Stewart and colleagues made an important advance in this field by showing that PCR-amplified linear dsDNA, flanked by short (42-bp) regions of homology to a plasmid (BOX 1), could be efficiently targeted to a plasmid by electroporating the dsDNA into *recBC sbcA* strains[6]. They also showed that, in doing so, a more flexible *E. coli* homologous recombination system was generated. *sbcA* is a mutation that activates expression of the *recE* and *recT* genes, which are encoded by part of the cryptic RAC PROPHAGE that is present in *E. coli* K12 strains[43–46]. Youming Zhang and colleagues[6] showed that *recE*- and *recT*-encoded recombination functions enable genomic DNA to be modified directly with PCR-generated linear dsDNA targeting cassettes, rather than by using targeting cassettes that carry long homologies generated by a multi-step process including subcloning into plasmids.

Cloning through *recE recT* (called ET cloning or RecET cloning) was initially studied in a *recA recBC sbcA* host so that the targeting cassette would not be degraded by the RecBCD nuclease[6]. However, many useful strains are *recBC*$^+$, including strains that are

RECA
RecA is central to recombination in *Escherichia coli*, and all organisms have RecA homologues. It allows two homologous DNAs to find each other, and to trade DNA strands by binding to a single-stranded region in one of the DNAs and by using that strand to search for its double-stranded DNA (dsDNA) homologue. It then binds to a homologue, causing the single strand to pair with its complement in the dsDNA, displacing the identical strand of the duplex and generating a key intermediate in the recombination process.

POSITIVE–NEGATIVE SELECTION
When the presence of a cassette is positively selected for, for example by drug resistance, and then negatively selected for, by eliminating cells that express a second selectable marker.

DH10B
A strain of *Escherichia coli* that has been modified and selected to accept large BAC clones by transformation. DH10B is defective for RecA recombination.

RAC PROPHAGE
Escherichia coli and other bacteria contain, in their chromosomes, remnants of viruses or prophages, such as Rac in *E. coli*, that often are defective and contain only a few genes of the original virus. Two Rac genes, *recE* and *recT*, encode homologous recombination functions, and are normally silent, but the *sbcA* mutation activates their constitutive expression.

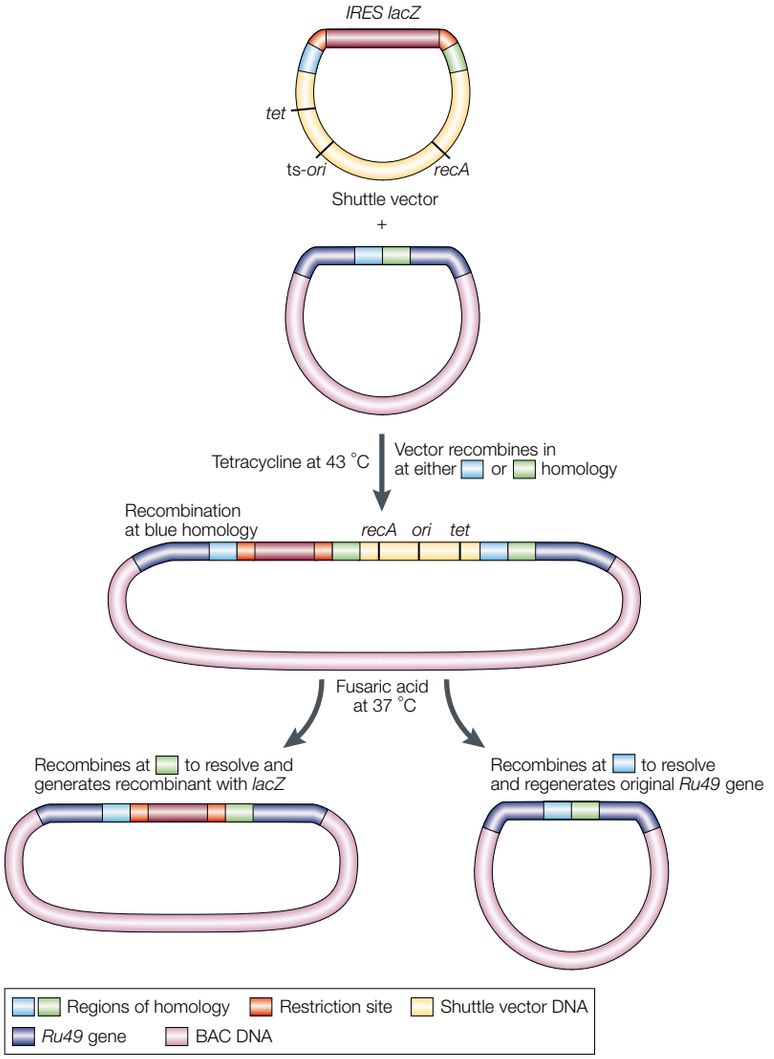


Figure 2 | **RecA-mediated recombination and gene modification.** The mouse *Ru49* zinc-finger gene is present on a 131-kb bacterial artificial chromosome (BAC) clone in *Escherichia coli*[41]. A vector that contains two cloned segments of the *Ru49* gene (~1 kb each, shown in dark blue) is made (not shown) and the *lacZ* cassette is cloned between these two segments. The vector carries the *recA* gene to provide recombination activity, *tet* for tetracycline resistance (Tet^R), and the temperature-sensitive origin of replication (ts-*ori*). At 43 °C, in the presence of tetracycline, only plasmids that have integrated into the BAC by recombining at the regions of homology in the BAC *Ru49* gene are maintained, as shown. Subsequent growth at 37 °C in the presence of fusaric acid selects for cells that have lost the Tet^R marker, in which the plasmid has recombined out of the BAC, through one homology segment. The net result of this integration/resolution reaction is a recombinant BAC that contains *lacZ* or the regeneration of the wild-type BAC. *IRES*, internal ribosome entry site.

commonly used for carrying P1, BAC or PAC plasmids. To allow ET cloning in $recBC^+$ strains, the pBAD-ETγ plasmid was developed[6] (FIG. 3a). pBAD-ETγ contains the *recE* gene under the control of the ARABINOSE-inducible pBAD promoter, the *recT* gene expressed from the constitutive EM7 promoter, and the bacteriophage-λ *gam* gene expressed from the constitutive Tn5 promoter. The addition of arabinose activates *recE* expression and establishes higher recombination activity in the cell. The *gam* gene of bacteriophage-λ was incorporated into pBAD-ETγ to inhibit RecBCD, thereby preventing the RecBCD-dependent destruction of the targeting cassette. Interestingly, in a $recA^-$ $recBCD^+$ host, pBAD-ETγ expression generated three times as many recombinants as it did in a $recA^+$ $recBC^-$ $sbcA^-$ host using a linear chloramphenicol resistance cassette to target the *Hoxa* region cloned on a P1 vector[6].

ARABINOSE
A simple five-carbon sugar metabolized by *Escherichia coli*, which is used as a chemical to induce and activate expression from the promoter pBAD.

λ-encoded Red system. Bacteriophage-λ also contains a homologous recombination system termed Red, which is functionally analogous to the RecET recombination system of Rac. Like RecET, Red recombination requires two genes: *redα* (or *exo*), which is analogous to *recE*, and *redβ* (or *bet*), which is analogous to *recT*. Exo is a 5′–3′ exonuclease that acts on linear dsDNA, as shown in FIG. 3c[47,48]. Beta binds to the ssDNA overhangs that are created by Exo and stimulates annealing to a complementary strand, but cannot promote strand invasion and exchange on its own[49,50]. The recombination functions of Exo and Beta are again assisted by bacteriophage-λ-encoded Gam, which inhibits the RecBCD activity of the host cell[51]. Kenan Murphy and colleagues have shown that λ-Red-mediated recombination events are 10–100 times more efficient than those observed in *recBC sbcBC* or *recD* strains[5,52]. Because homologous recombination is increased by introducing phage-encoded protein functions to the host, this procedure is applicable to any *E. coli* strain and to other bacterial species as well.

Red recombination with a plasmid system. To facilitate BAC modification using λ-Red, a derivative of pBAD-ETγ was constructed, called pBAD-αβγ. In pBAD-αβγ, *exo* is under the control of the promoter pBAD, and *bet* and *gam* are expressed constitutively from the EM7 and Tn5 promoters, respectively. pBAD-αβγ is one- to threefold more efficient at BAC recombination than pBAD-ETγ[8]. Like pBAD-ETγ, pBAD-αβγ promotes homologous recombination independently of *recA*, and induction with arabinose controls the time period over which λ-Red recombination levels are high in the cell, which, in the absence of RecA, reduces the risk of unwanted intramolecular recombination in the BAC.

Following on from the development of RecET/Red recombination[9], other labs have explored ways in which to regulate and express the three phage proteins — Gam, Beta and Exo — to maximize efficient dsDNA-break-induced recombination. As discussed below, Daiguan Yu and colleagues[7] have coordinately expressed the three phage genes from their natural location on a defective λ-prophage, whereas, Kirill Datsenko and Barry Wanner[53] have expressed them from a low-copy plasmid under pBAD control.

Red recombination through defective prophages. In the defective λ-prophage-based system[7], *gam*, *bet* and *exo* are expressed from a λ-prophage that is integrated into the *E. coli* chromosome (FIG. 3b), and their expression is under the tight control of the temperature-sensitive λ-*cI857* repressor. At 32 °C, when the repressor is active, the expression of these genes is undetectable.

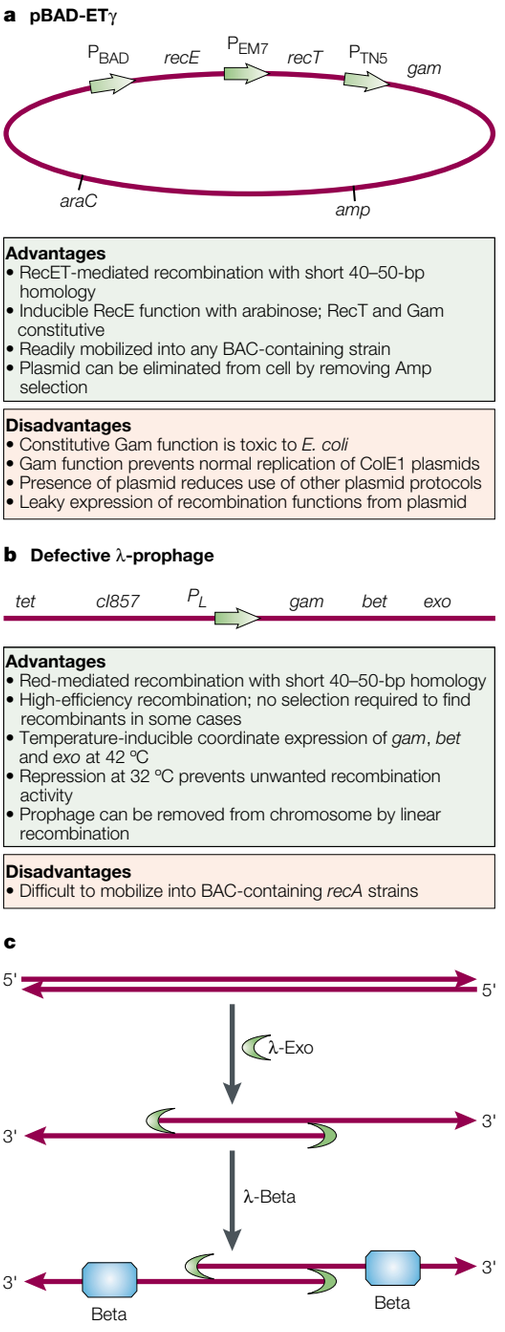


Figure 3 | **A comparison of phage-mediated expression systems.** The structure, advantages and disadvantages of the expression plasmid, pBAD-ETγ, and the defective λ-prophage are shown. **a** | In pBAD-ETγ, which is used in ET cloning[6], *araC* encodes the repressor of the pBAD promoter, but in the presence of arabinose, *araC* acts as a positive regulator of this promoter. Other configurations of this vector have also been made[8,53,60,62]. **b** | The defective prophage is integrated into the *E. coli* chromosome of the DY380 strain and expresses *exo*, *bet* and *gam* under the control of the P_L promoter and the temperature-sensitive repressor, *cI857*. Genes that encode CRE and FLPE are present on other derivatives of DY380 (REF. 56) and replace the tetracycline-resistance gene (*tet*) as shown. **c** | The activities of the bacteriophage-λ-encoded proteins, Exo and Beta. Double-stranded DNA (dsDNA) ends are attacked by the exonuclease, λ-Exo, which digests 5′ DNA ends to leave 3′ single-stranded DNA overhangs. Beta binds to these overhangs and anneals them to complementary ssDNA in the cell. RecE is an analogue of λ-Exo, and RecT is an analogue of λ-Beta. Only the orthologous pairs of functions are active *in vivo* for recombining linear dsDNA to its target[77].

However, when cells are shifted to 42 °C for 15 min, the repressor is inactivated and the genes are coordinately expressed from the λ-*pL* promoter at very high levels, making it possible to achieve greater than 10^4 recombinants per 10^8 electroporated cells[7].

Expression of *gam* causes a RecBCD defect, a condition that results in plasmid instability and loss of cell viability[54,55]. The tight repression of all three genes afforded by the prophage system prevents any *gam* expression. The prophage itself is genetically stable and so does not rely on the presence of drug selection for maintenance.

The defective λ-prophage has also been transferred to the BAC host strain DH10B so that it can be used for recombineering BACs[56]. The modified DH10B strain, called DY380, can be transformed with BAC DNA at efficiencies of 10^{-6} to 10^{-4} per electroporated cell. The use of DY380 cells for BAC recombineering has been recently shown[56]. A 250-kb mouse BAC that contains the neuronal-specific enolase 2 (*Eno2*) gene was introduced into DY380 cells by electroporation. The BAC was then modified by introducing a *cre*-expressing targeting cassette into the 3′ end of the *Eno2* gene using Red recombination[56]. Using the general approach shown in FIG. 1, correctly targeted colonies were obtained at an efficiency approaching 10^{-4} per electroporated cell after the induction of Red expression; no targeted colonies were obtained in uninduced cells.

The modified full-length BAC was injected into mouse zygotes to generate a transgenic mouse line[56]. Two other transgenic lines that carry a shorter 25-kb subclone of the modified *Eno2* gene on a plasmid were also established as controls. The 25-kb subclone carries the entire modified *Eno2*-coding region, 10 kb of 5′-flanking sequence and 5 kb of 3′-flanking sequence. The activity of the *cre* gene in the different transgenic lines was then assessed by crossing the mice with Rosa26 reporter mice, which carry a *lacZ* reporter gene that can be activated by Cre recombinase[57]. In mice that carry the full-length BAC transgene, Cre activity was detected in all *Eno2*-positive neurons. By contrast, not all *Eno2*-positive neurons expressed *cre* in the transgenic mice that carried the smaller 25-kb subclone, and *cre* expression varied between the two different control lines[56]. These results are consistent with previous studies that have shown that regulatory sequences can be located hundreds of kilobases from a gene[58], and highlight the usefulness of BAC engineering for generating *cre*-expressing lines for use in the conditional inactivation of gene expression.

Arabinose-inducible Flpe[59] or *cre* genes have also been introduced into the defective prophage that is carried in strain DY380 (REF. 56). The site-specific recombinases Flpe and Cre are important tools that are used to add or delete DNA segments. In these derivatives of DY380, the expression of Flpe and Cre can be induced by arabinose to remove a selection marker that is flanked by the site-specific recognition sites of the recombinase — *FRT* or *loxP*, respectively. This is important in cases in which the selection marker interferes with the expression of the targeted locus (for more, see the accompanying review by Mark Lewandoski on p743 of this issue).

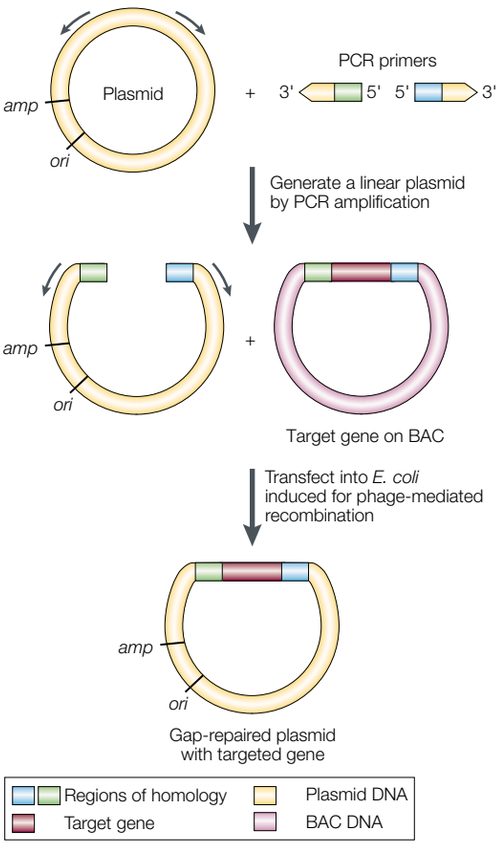


Figure 4 | **Retrieving cloned DNA by gap repair.** Retrieving mouse genomic DNA of up to 80 kb from bacterial artificial chromosomes (BACs) into PCR-amplified vectors has been done using recombineering techniques[56]. Only the plasmid is linearized and transformed into a recombination-competent cell, which contains the BAC[62]. Recombination occurs between homologies on the end of the linear vector and the BAC DNA. This method eliminates standard cloning technology and, importantly, the cloned segment is not replicated *in vitro*, reducing the chances of extraneous changes occurring in the sequence.

CRE
Cre is a site-specific recombinase that recognizes and binds to specific sites called *loxP*. Two *loxP* sites recombine at nearly 100% efficiency in the presence of Cre, allowing DNA cloned between two such sites to be removed by Cre-mediated recombination.

FLPE
Flpe is a genetically enhanced, site-specific Flp recombinase that recognizes and binds to *FRT* sites.

DY380
A derivative of DH10B. The defective λ-prophage, used to express the *red* and *gam* functions, has been moved into the chromosome of this strain.

SACB
sacB encodes the SacB protein, which converts sucrose into a toxic form that kills bacteria. This can be used in negative selection for the *sacB* gene.

FLAG TAG
A short peptide sequence that is added to a protein to allow the protein to be recognized by antibodies raised against the flag tag.

Modifying BACs: no markers left at target site

Selection/counterselection with SacB. A two-step procedure for BAC targeting has been developed that allows many kinds of mutation to be introduced into BACs without leaving behind a drug-selectable marker at the targeted locus[60]. In this two-step procedure, a PCR-generated targeting cassette that contains a *SACB–neo* fusion gene is targeted to a BAC or other DNA[49]. Cells that contain the *sacB–neo* cassette, which has been targeted to the genomic DNA of the BAC, are selected on the basis of their neomycin resistance and are then transformed with a second targeting DNA to the same region. This second cassette is designed to replace the entire *sacB–neo* cassette and might contain short genomic sequences that carry a more subtle mutation, such as a small insertion. Placing these newly transformed cells on media with 7% sucrose kills the cells that still express *sacB*[61]. Because spontaneous mutations occur in *sacB* to cause sucrose resistance at frequencies approaching 1 in 10^4, recombinants are identified among sucrose-resistant colonies as those that have also become neomycin sensitive. By combining the power of RecET recombination with selection/counterselection using *sacB–neo*, other kinds of genetic change apart from insertions can be generated, including deletions and point mutations. These mutations can be introduced into virtually any large DNA molecule, such as a BAC, PAC or the *E. coli* chromosome, without the accompanying selectable markers.

Direct BAC or genome modification. The high frequency of recombination that is generated by the defective prophage system (FIG. 3) has allowed the bacterial genome or BACs to be modified in a single step without drug selection. E.-Chiang Lee and colleagues[56] directly introduced a 24-bp sequence, by recombination and without selection, into the 5′-end of the SRY-box containing gene 4 (*Sox4*), which was present on a 125-kb BAC. This 24-bp sequence encoded a FLAG TAG. For these experiments, a 114-bp targeting cassette was generated in which two 45-bp arms, homologous to the *Sox4* gene, flanked the 24-bp flag tag sequence. The cassette sequence was created from two 69-bp oligonucleotides that were complementary at their 3′ ends to the 24-bp tag sequence. These two overlapping oligonucleotides were annealed to each other and filled in by Taq polymerase, to give rise to the targeting cassette. After the introduction of this cassette by electroporation into induced, recombination-competent DY380 cells that carry the 125-kb BAC, recombinant colonies that contain the flag tag were identified by colony hybridization using a flag-tag-specific probe. The overall targeting frequency of this experiment was 1.7×10^{-3}.

Because the homology segments that are required for RecET/Red-mediated recombination can be very short, targeting cassettes can be made by simply annealing together two complementary, synthetic ssDNA oligonucleotides. Yu and colleagues have shown that a 70-bp targeting cassette constructed in this manner recombines with the *E. coli* chromosome to create point mutations at frequencies that approach 1 in 1,000 electroporated cells[7]. Point mutations that correspond to human disease-causing mutations can therefore be introduced into any human gene that is carried on a BAC to assay their effect *in vivo*, alone or in combination with other mutations.

Cloning DNA by gap repair

Subcloning DNA from BACs. DNA fragments can also be subcloned from BACs by RecET/Red-mediated recombination, without the use of restriction enzymes or DNA ligases. So, any region of the BAC is amenable to subcloning, without an investigator having to rely on the placement of appropriate restriction enzyme sites[56,62]. Subcloning relies on gap repair to recombine the free ends of a linear plasmid vector with homologous sequences carried on the BAC (FIG. 4). Two types of plasmid replicon — p15A and ColE1 — have been used successfully in this type of gap-repair cloning[56,62]. The linear plasmid vector, with, for example, an

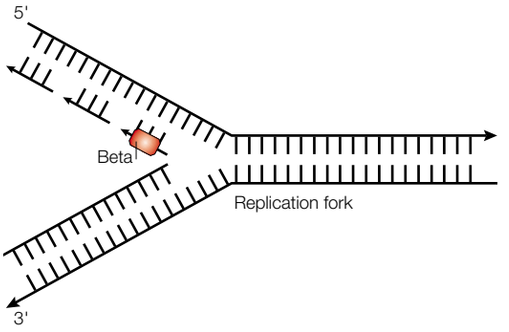


Figure 5 | **Recombination of ssDNA into the genome.** When single-stranded DNA (ssDNA) is electroporated into cells, it is bound by Beta and recombines into the genome or into a bacterial artificial chromosome by homologous recombination. Evidence indicates that Beta-bound ssDNA anneals to its ssDNA complement at the replication fork. The strand of DNA that corresponds to that made by lagging-strand synthesis is the most recombinogenic of the two replicated strands, indicating that Beta might anneal the ssDNA to a gap caused by DNA replication[64].

amp-selectable marker and an origin of replication, carries the recombinogenic ends. The vector is generated by PCR amplification using two chimeric primers. The 5′-end of each primer has homology to the extremities of the BAC sequence to be subcloned; the 3′-end of each primer is used to prime and amplify the linear plasmid DNA. Recombination generates a circular plasmid in which the DNA insert is retrieved from the BAC through gap repair. Circular recombinant plasmids are selected by their Amp^R phenotype. The size of the fragment that can be subcloned is dependent on the copy number of the cloning vector. With a high-copy vector, such as the ColE1-derived pBluescript, fragments larger than 25 kb are difficult to subclone[56]. However, with a lower-copy vector, such as the ColE1-derived pBR322, a fragment as large as 80 kb has been subcloned[56].

The linear vectors used for gap repair can generate circular plasmids in which the two ends of the vector seem to simply join together. Youming Zhang *et al.*[62] have shown that this 'end joining' is caused by very short homologies, six nucleotides or longer, near each end. If such homologies are introduced, they cause a background among drug-resistant clones that must be distinguished from recombinants. Normally, the percentage of these 'end-joined' products is less than 20% of cloned recombinants[62].

Cloning DNA from complex mixtures. Following the lead of earlier studies in yeast[63], gap repair can also be used to directly subclone sequences from purified total genomic DNA using the RecET recombination functions[62]. As expected, it is more difficult to subclone fragments from mouse genomic DNA than from less complex DNAs, such as yeast or *E. coli* genomic DNA. This is thought to reflect the physical limitation imposed by co-electroporation of the linear cloning vector with total genomic DNA[62]. With more complex genomes, fewer cells will be co-electroporated with both the targeting vector and the target. So far, all experiments that report the subcloning of DNA from complex mixtures have used plasmid-based expression systems for producing the recombination proteins, and only small fragments (4.5 kb) have been subcloned. It will be interesting to examine the effectiveness of the defective prophage-based Red recombination system in retrieving DNA from such complex DNA mixtures.

Recombineering using ssDNA

Recombineering can also be done using single-stranded oligonucleotides as the targeting cassette. In addition to being simpler to construct, such single-stranded oligos are 10–100-fold more efficient than recombineering with dsDNA; up to 6% of electroporated cells can be recombinant[7,64]. These and other features discussed below make this technology very useful for modifying BAC DNA[65].

In *E. coli*, the *galK* gene encodes the bacterial galactokinase, which is required for metabolizing galactose. Using ssDNA oligos, a single base change has been introduced into *galK* and a 3.3-kb insertion in *galK* has been removed. Single-stranded oligos have also been used to cure five different *Tn10* insertions at different places on the *E. coli* chromosome[64]. Whereas Exo, Beta and Gam are required for dsDNA recombination, only Beta is required for ssDNA recombination[64]. Maximum recombination requires oligos of 70-nucleotides in length, although 40–60-nucleotide-long oligos are only fivefold less efficient[64].

Strand bias. Recombination with either of two complementary DNA oligos has shown that one DNA strand is more competent for recombination than the other[64]. This strand bias has been examined at several positions around the bacterial chromosome, revealing that the preferred strand for recombination correlates with the lagging strand of DNA replication at each site tested. These results indicate that this strand bias might be associated with the direction of DNA replication at the region being targeted, and that ssDNA recombination occurs near the replication fork[64]. DNA replication results in transient regions of ssDNA that might be accessible to the Beta-mediated annealing of the ssDNA oligo. DNA polymerase and DNA ligase could then complete the joining of the annealed oligo to the lagging strand (FIG. 5). The increased recombination efficiency of the lagging-strand oligos might reflect the increased frequency of single-stranded regions during lagging- versus leading-strand synthesis.

ssDNA recombination might occur through a different mechanism than dsDNA recombination. ssDNA recombination might require only the annealing of one single-stranded oligo to single-stranded regions in the replicating target DNA. ssDNA recombination also occurs in yeast with a strand bias that might also be dependent on replication[66,67]. The yeast functions that are required for this recombination are, however, unknown, which makes the finding that only the Beta function from bacteriophage-λ is required in *E. coli* that much more significant.

BAC modification using ssDNA. A point mutation has been engineered into the mouse *Brca2* (breast cancer susceptibility 2) gene that is carried on a BAC using a 70-nucleotide oligo[65]. The targeting efficiency was several times higher than that observed using dsDNA made from annealed oligos, and at least 50 times higher than that observed using dsDNA generated by PCR and containing large regions of non-homology at its centre[7,56,65]. Oligos that are 140 nucleotides long have also been used to introduce a 29-amino-acid in-frame deletion into exon 11 of the *Brca2* gene on a BAC, and a 1.93-kb deletion into the BAC vector backbone[65]. Finally, a 164-nucleotide oligo has been used to introduce a 24-bp flag tag into the 5′-end of *Brca2* (REF. 65). The targeting efficiency for the 164-nucleotide flag tag oligo was nearly the same as that for generating deletions using the 140-nucleotide oligo. In both cases, recombinant frequencies approach 1% of electroporated cells. These ssDNA recombineering experiments were all done in DY380 derivatives that express *gam*, *bet* and *exo*. Similar experiments should be possible in BAC-containing strains that express only the Beta ssDNA-binding function that is required for this recombination.

Future directions

Genetic engineering can now be accomplished in *E. coli* without using restriction endonucleases and DNA ligase. RecET/λ Red homologous recombination can efficiently modify chromosomal or plasmid DNA *in vivo* by recombination with linear ssDNA or dsDNA electroporated into a cell. The most important aspects of recombineering are that only short homology segments are required to direct the recombination, and recombination efficiency rates allow recombinants to be screened rather than selected. The fact that recombinants can be screened means that only one recombination step is required to create the desired modification, and that modification is created directly, without selective markers being added to the BAC.

These homologous recombination systems enable large fragments of genomic DNA to be modified or subcloned in a way that was unimaginable only a few years ago and have extended the spectrum of applications for homologous recombination beyond those possible in yeast. The high frequency of recombination offered by these plasmid and phage systems allows BAC DNA to be manipulated without drug selection. Virtually any kind of mutation can now be engineered into a BAC in the absence of drug-selectable markers or of *loxP* or *FRT* sites, the presence of which can affect the function of the region of the BAC being studied.

These recombination systems also simplify the generation of complicated transgenic and knockout constructs for use in gene-function studies[56,68]. They also make it possible to introduce *cre*- or *FLP*-expressing genes into BACs, thereby facilitating the generation of *cre*- or *FLP*-expressing transgenic lines for use in conditional knockout studies. Genes on BACs can also be targeted by fusion sequences that encode epitope markers or GFP, and the BACs can then be introduced into the germ line of mice. Likewise, a gene carried on a BAC can be replaced with a special allele, and the function of the 'knock-in' mutation can then be assayed in transgenic mice.

The ability to subclone large segments of genomic DNA and to make well-defined deletions in BACs[52,56,62,65,69] will also aid in defining and studying regulatory elements or locus control regions that can be located far from the genes that they control. We expect that many such elements will be identified over the next few years by comparative genome sequencing.

Similarly, Beta-mediated ssDNA recombination provides new and exciting avenues for studying and modifying the genomes. Because oligo-mediated recombination occurs in both *E. coli* and yeast, it is possible that recombination through the annealing of ssDNAs might occur in a wide range of organisms. So, recombineering with ssDNA might be applicable to higher eukaryotes by mechanisms as simple as overexpressing Beta (or a functionally similar ssDNA annealing protein), or by introducing the protein bound to ssDNA during electroporation. Potentially, this method could be used to directly introduce mutations into embryonic stem cells, which removes the need for constructing targeting vectors. Likewise, mutations in diseased cells could be corrected using this technique, providing a new method for gene therapy. In addition, because oligos are so efficiently incorporated *in vivo*, the potential also exists to introduce special chemical adducts and modifications from the oligos directly into the chromosome, therefore providing special 'tags' in the DNA of living cells.

Thirty years ago, restriction enzymes were discovered that led to revolutionary changes in molecular biology and to the advent of genetic engineering. However, homologous recombination is a more precise, efficient and versatile means of engineering DNA molecules. The precision with which recombinant DNA molecules can be created using restriction enzymes and DNA ligase is limited by the availability of unique and correctly positioned restriction sites. Homologous recombination, conversely, can generate recombinant molecules without the need for unique or special sites. During the next few years, as the genomes of most model organisms are sequenced, these recombination-based approaches will become all the more powerful, and could even make restriction enzymes and DNA ligase obsolete in future genetic engineering studies.

Although many uses for recombineering in *E. coli* are already apparent, it is likely that we have only scratched the surface of what is possible. After all, the first papers that described the recombineering of BACs were published less than two years ago. As with PCR amplification, it will no doubt be many years before the power of this new technology is fully evident. It would be interesting to have a crystal ball with which to see where recombineering will take us.

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Brca2 | *Eno2* | *gata2* | *Ru49* | *Sox4*

FURTHER INFORMATION

***E. coli* genetic stock centre**:
http://cgsc.biology.yale.edu/top.html

ET cloning: http://www.embl-heidelberg.de/ExternalInfo/stewart/ETcloning-textonly.html

HGMP BAC, PAC and YAC resources:
http://www.hgmp.mrc.ac.uk/Biology/resources_index.html

RecA web site:
http://www.tigr.org/~jeisen/RecA/RecA.html

Research Genetics BAC and PAC resources:
http://www.resgen.com/intro/libraries.php3