The univector plasmid-fusion system, a method for rapid construction of recombinant DNA without restriction enzymes

Qinghua Liu*†‡, Mamie Z. Li*†‡, Deborah Leibham*, David Cortez* and Stephen J. Elledge*†§

Background: Modern biological research is highly dependent upon recombinant DNA technology. Conventional cloning methods are time-consuming and lack uniformity. Thus, biological research is in great need of new techniques to rapidly, systematically and uniformly manipulate the large sets of genes currently available from genome projects.

Results: We describe a series of new cloning methods that facilitate the rapid and systematic construction of recombinant DNA molecules. The central cloning method is named the univector plasmid-fusion system (UPS). The UPS uses Cre–lox site-specific recombination to catalyze plasmid fusion between the univector — a plasmid containing the gene of interest — and host vectors containing regulatory information. Fusion events are genetically selected and place the gene under the control of new regulatory elements. A second UPS-related method allows for the precise transfer of coding sequences only from the univector into a host vector. The UPS eliminates the need for restriction enzymes, DNA ligases and many in vitro manipulations required for subcloning, and allows for the rapid construction of multiple constructs for expression in multiple organisms. We demonstrate that UPS can also be used to transfer whole libraries into new vectors. Additional adaptations are described, including directional PCR cloning and the generation of 3′ end gene fusions using homologous recombination in Escherichia coli.

Conclusions: Together, these recombination-based cloning methods constitute a new comprehensive approach for the rapid and efficient generation of recombinant DNA that can be used for parallel processing of large gene sets, a feature that will facilitate future genomic analysis.

Background

The discovery of restriction enzymes in the early 1970s led to a revolution in the analysis of nucleic acids [1,2]. It soon became apparent that the defined ends resulting from the cleavage of DNA by such enzymes could be joined by DNA ligases, thus allowing the recombination of DNA molecules in vitro [3]. Together with the discovery of autonomously replicating plasmids, this made possible the isolation, propagation, and purification of individual fragments of DNA. These discoveries ushered in the era of molecular cloning in biological research. The ability to recombine DNA fragments led not only to the cloning of DNA fragments that encoded genes, but also to the manipulation of genes in such a way as to alter their regulatory sequences [4]. The coding regions of genes could be placed under the control of regulated promoters and reintroduced into organisms to explore the consequences of altered regulation. Coding regions could also be fused to other coding regions to produce hybrid proteins with unique properties that could be exploited for genetic or biochemical purposes.

The manipulation of genes has grown more sophisticated as new techniques have emerged allowing finer and finer alterations of sequences. The number of plasmid vectors bearing unique properties for the analysis of genes has also grown. For a routine analysis of a new gene it might be desirable to express it in bacteria as a glutathione-S-transferase (GST) fusion protein or with a six-histidine (His6) tag for purification and antibody production, to fuse it to the DNA-binding domain of the yeast and bacterial transcription factors Gal4 or LexA for yeast two-hybrid interaction screening, to express it from the T7 promoter to allow generation of a riboprobe or mRNA for in vitro transcription and translation, and express it in baculovirus, all in the course of a single study. One might also wish to express a particular gene under the regulation of different promoters in a variety of organisms or to mark it with different epitope tags to facilitate subsequent biochemical or immunological analysis. All of these manipulations consume significant amounts of time and energy for two reasons. First, each of the different vectors required for these studies were, for the most part, developed independently and thus contain

Addresses: *Howard Hughes Medical Institute, †Verna and Marrs McLean Department of Biochemistry, ‡Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA.

*Q.L. and M.Z.L. contributed equally to this work.

Correspondence: Stephen J. Elledge
E-mail: univ@bcm.tmc.edu

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different sequences and restriction sites for the insertion of the genes under study. Genes must therefore be individually tailored to adapt to each of these vectors. Secondly, the DNA sequence of any given gene varies and can contain internal restriction sites that make it incompatible with particular vectors, thereby complicating manipulation. The advent of the polymerase chain reaction (PCR) has greatly facilitated the alteration of gene sequences and the creation of compatible restriction sites for subcloning purposes. The high error rate of thermostable polymerases requires that the sequence of each PCR-derived DNA fragment must be verified, however, and this clearly represents a time-consuming process.

The availability of whole genome sequences now presents us with the opportunity to analyze large sets of genes for both genetic and biochemical properties. The need to perform parallel processing of large gene sets exponentially amplifies the inherent defects associated with conventional cloning methods. In this paper, we describe a series of recombination-based approaches that significantly reduce the time and effort involved in generating recombinant DNA for gene analysis and cDNA library construction. We have developed a method, termed the univector plasmid-fusion system (UPS), whereby a gene in a particular type of plasmid — the univector — can be placed under the control of any of a variety of promoters or fused in-frame to other coding sequences without the use of restriction enzymes. This system, together with the additional methods described herein, circumvents the problems associated with conventional restriction-enzyme-mediated cloning methods and provides a multi-faceted approach for the rapid and efficient generation and manipulation of recombinant DNA, thus making possible the parallel processing of whole genome sets of coding sequences.

Results

Development of the UPS

UPS is based upon plasmid fusion using the Cre–lox site-specific recombination system of bacteriophage P1. The product of the cre gene is a site-specific recombinase that catalyzes recombination between two 34 bp loxP sequences and is involved in the resolution of P1 dimers generated by replication of circular lysogens [5]. Cre can function in vivo in several organisms including bacteria, fungi and mammals and also functions in vitro [6–8]. The scheme for UPS is shown in Figure 1a. The pUNI plasmid is the univector into which the gene of interest must be inserted. The pHOST plasmid is the recipient vector containing the appropriate transcriptional regulatory sequences that will eventually control the expression of the gene of interest in the designated host cells. It may also carry translational initiation and additional coding sequences for generating fusion proteins. A recombinant expression construct is made through Cre–loxP-mediated site-specific recombination that fuses pUNI and pHOST.

The univector has a number of properties that facilitate its use in this system: the maps for several different univector plasmids are shown in Figure 1d. General features include a loxP site placed directly adjacent to the 5’ end of the polylinker for insertion of cDNAs. The loxP sequence has a single open reading frame (ORF) that is in-frame with the ATG of the NdeI and Nol sites of the polylinker. This facilitates the subsequent generation of protein fusions as noted below. Following the polylinker are bacterial and eukaryotic transcriptional terminators to facilitate 3’ end formation of transcripts. The univector also has a conditional origin of replication derived from the plasmid R6Kγ that allows its propagation only in bacterial hosts expressing the pir gene (encoding the essential replication protein π) originally from R6Kγ [9]. The univector contains the neo gene (which encodes kanamycin resistance, KnR) from the transposable element Tn5 for selection in bacteria. The pUNI20 univector contains additional site-specific recombination sites, such as RS, which facilitate precise ORF transfer (POT). The pUNI30 univector contains half of a lacO site for directional PCR cloning (see below).

The pHOST vector features include the ColE1 origin of replication, the bla gene (which encodes ampicillin resistance, Ap8) for propagation and selection in bacteria, a loxP site for plasmid fusions and a specific promoter residing upstream of, and immediately adjacent to loxP. Host vectors may also contain sequences responsible for propagation, selection and maintenance in organisms other than Escherichia coli. In pHOST vectors intended for protein fusions, loxP is placed in the same reading frame as the additional coding sequence. In order for all UPS-derived protein fusions to be automatically in-frame, the target gene must be cloned into the univector in-frame with the loxP ORF (Figure 1d).

The UPS reaction can be carried out both in vitro or in vivo in the presence of Cre recombinase. The reaction results in the fusion of the pUNI plasmid with a pHOST plasmid via the loxP site to create a recombinant plasmid, thereby placing the target gene under the control of the regulatory sequences on the host vector. Selection for the recombination products of UPS is achieved by selecting for kanamycin resistance after transformation into a pir– strain (typical E. coli strains are pir+) because the neo gene in pUNI can only be propagated when covalently linked to an origin of replication (oriColE1) in pHOST that is functional in a pir– background.

Generation of a functional GST–Cre fusion protein

To simplify Cre purification, a plasmid, pQL123, expressing a GST–Cre fusion protein was constructed. The expression of GST–Cre was highly induced in E. coli in
Figure 1

(a) A schematic representation of the UPS cloning strategy. Cre catalyzes site-specific recombination between loxP sites, generating plasmid fusion between pUNI and pHOST. This results in a fusion plasmid in which the gene of interest from pUNI is placed under the control of pHOST regulatory elements. (b) Expression and purification of GST–Cre. Lane 1 contains 100 µg total cell extract from GST–Cre-expressing BL21/pQL123 cells grown in the presence of IPTG. Lane 2 shows GST–Cre (2 µg) after a single-step purification with glutathione-Sepharose beads [10] (Figure 1b). GST–Cre retained high recombinase activity as measured by UPS.

(b) GST–Cre Number of ApR Number of KnR KnR/ApR

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(c) GST–Cre (µg) Number of ApR Number of KnR

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(d) Maps of pUNI10, pUNI20 and pUNI30. Nucleotide positions of restriction sites are shown with non-unique sites in bold. Functional sequences are shown as filled boxes and are labeled inside the circle. Below each map is the polylinker sequence displayed as codons in-frame with the QRF of loxP.

The presence of isopropyl β-D-thiogalactoside (IPTG) and was purified to near homogeneity with glutathione–Sepharose beads [10] (Figure 1b). GST–Cre retained high recombinase activity as measured by UPS. The efficiency of this reaction reached up to 16.8% (Figure 1c), similar to that for native Cre [6]. The efficiency of GST–Cre-mediated recombination was examined in a second reaction which involved the production of an GST-tagged version of the Saccharomyces cerevisiae ubiquitin–ligase protein Skp1 (shown in Figure 2a) [11]. Recombinant plasmids isolated from KnR transformants were shown by restriction analysis to be correct fusion products of the univector and the host vector via the loxP sites. In this case, 10 out of 12 KnR transformants contained the desired fusion of the two plasmids (Figure 2b). Two of the transformants (Figure 2b, lanes 8,10) contained two copies of pHOST fused to one copy of the pUNI construct: it should be noted that these fusion products also have a correct fusion junction and are fully functional for most needs. This method is highly efficient and typically requires analysis of only one or two clones to identify the desired construct.
Expression of UPS-derived constructs in multiple systems. (a) A schematic representation of the generation of epitope-tagged expression constructs by UPS. (b) A 
PstI restriction analysis of the UPS-derived GST-lox-Skp1 recombinant plasmids. Plasmid DNA from 12 independent KnR transformants was digested with PstI and the digestion products were separated on a 1% agarose gel and visualized by ethidium bromide staining (lanes 1–12). The parental plasmids, pUNI10-SKP1 (pQL138; lane P1), and pHB2-GST (lane P2), each contain a single PstI restriction site and upon cleavage generate a 2.8 kb or 5.0 kb fragment, respectively. The correct fusion product produces two bands of 2.0 kb and 5.8 kb. (c,d) Expression levels of GST–Skp1 constructs generated by UPS or conventional methods. Proteins prepared from a conventionally-derived GST–Skp1 plasmid (lanes 1,2) and three independent UPS-derived GST-lox–Skp1 plasmids (lanes 3–8) were separated by SDS–PAGE and stained with Coomassie blue or immunoblotted with anti-Skp1 antibodies. The arrow indicates the position of GST–Skp1, whereas the arrowhead indicates the position of slightly larger GST–lox–Skp1 protein. The asterisk denotes a degradation product. Approximate expression levels of UPS-derived constructs in S. cerevisiae and comparison between loxP- and loxH-containing constructs. Protein extracts were prepared using a standard protocol. Vector alone (pQL138; lane 1), conventionally-derived control GAL:MYC3–RNR4 (pQL222) lacking a lox site (lane 2), UPS-derived GAL:MYC3–RNR4 constructs containing loxP (lane 3) or loxH (lane 4); vector alone (pHY314-MYC3; lane 5), or GAL:MYC3–RAD53 (pUNI-RAD53; pHY314-MYC3) made by UPS (lane 6). Western blots were probed with anti-Myc antibodies for expression and anti-Skp1 antibodies for a loading control in lanes 1–4.

Testing expression constructs made by UPS

A series of expression plasmids were made by UPS and tested for expression in several contexts. The SKP1 ORF in pUNI10 was fused to the pHB2-GST recipient vector by UPS to create a bacterial GST–lox–Skp1 fusion protein expressed from the E. coli tac promoter (Figure 2b). A similar GST–Skp1 expression plasmid lacking loxP (pCB149) made by conventional cloning was used as a control. Approximately equal amounts of the two fusion proteins were expressed (Figure 2c,d), indicating that the presence of loxP did not significantly affect either the transcription or the translation of the fusion protein.

The loxP sequence contains a potential 13 bp stem-loop structure, and secondary structures formed within the 5′ untranslated region (UTR) of an mRNA can potentially interfere with the initiation of translation in eukaryotic cells [12]. To test the effects of loxP sites upon translation, we made a series of lox sites containing mutations designed to reduce the stability of the stem-loop. These, together with a control site lacking the stem-loop, loxS (ATAACTCCG-TATAGCATACATT TAACCTGCATT; mutations in bold), were placed between the GAL1 promoter and a lacZ reporter gene and β-galactosidase expression was measured using S.cerevisiae Y80 as host. The loxP sequence reduced β-galactosidase expression fourfold relative to loxS. In
contrast, variousloxP mutants with decreased stem-loop
stability tended to result in better expression of β-galactosi-
dase. One mutant,loxH (ATTACGCTACATAGCAT-
CATTATCGAAGTTAT; mutations in bold), failed to
display inhibitory effects when compared withloxS (data
not shown). The effects ofloxH were further tested by
using it to place the gene encoding the ribonucleotide
reductaseRnr4 protein tagged with three copies of the Myc
epitope,MYC3-RNR4, under the control of theGAL1 pro-
* motor,loxH showed improved translational ability relative
toloxP (Figure 2e, compare lanes 2–4). It should be noted
that the control lacking alox site in Figure 2e has a differ-
t space and is not completely isogenic for the purposes
of comparing translational efficiency ofloxH. When paired
withloxP,loxH also retained 25% of the wild-type recombi-
national efficiency (data not shown) which is well within
the useful range for UPS-mediated plasmid construction.
We recommend thatloxH be used in recipient vectors
intended for transcriptional fusions to maximize expres-
sion, whereasloxP should be used for all other applications
because of its higher recombination efficiency.

Multiple genes have been tested using UPS and
expressed in several different organisms. In addition to
GST–Skp1 expression in bacteria, Myc3-tagged versions
ofRnr4 and the checkpoint proteinRad53 have been
expressed inS. cerevisiae(Figure 2e). Furthermore, many
baculovirus expression constructs have been made by
UPS and tested (our unpublished observations) and
shown here are GST–Rad53, MYC3–Rad53, and
HA3–Rad53 (Rad53 tagged at the amino terminus with
three copies of the hemagglutinin (HA) tag; Figure 2f).
The UPS-derives construct encoding GST–Rad53
exposed to the same level as GST–Rad53 generated
by conventional methods (Figure 2f, compare lanes 1 and
2). In mammals we have demonstrated expression of a
HA3-tagged F-box protein under the control of the
cytomegalovirus (CMV) promoter in Hela cells
(Figure 2g). Over 200 UPS-derives constructs show
expression success rates indistinguishable from those of
conventional cloning methods for all genes and all organ-
isms tested thus far.

**In vivo UPS**

Cre–loxP-mediated plasmid fusion can also occur in vivo.
Ideally, it would be desirable to have Cre present only tran-
siently to catalyze the initial fusion event, then absent to
allow the stable propagation of the recombinant products.
We therefore explored UPS in vivo in theE. coli strain
BUN13 which expresses an integrated cre gene under the
control of the lac promoter and in a second strain, BUN14,
carrying lac–cre on a plasmid, pQL269, with a temperature-
sensitive origin of replication derived from pS克莱01. Experi-
ments using BUN13 and co-formation of pUNI10 and
pQL103, a recipient plasmid, showed that the UPS reaction
occurs efficiently in vivo but many colonies had a mixture of
plasmids that required retransformation into a strain that did
not express cre to allow for stabilization (data not shown).
Results with BUN14 were better, however. Competent
cells were prepared from BUN14 cells grown at 42°C to
allow both Cre production and loss of pQL269. Co-tran-
formation of pUNI10 and pQL103 into these cells followed by
kanamycin selection at 42°C revealed that 25% of transfor-
mants contained the desired single pUNI10–pQL103 co-
tegnant. These experiments demonstrate that UPS can be
performed in vivo and provide an alternative to the in vitro
reaction when GST–Cre is not available.

**UPS can be used for precise ORF transfer (POT)**

UPS-derived constructs are suitable for the vast majority
of expression needs. In rare cases, however, where the size
of the recombinant molecule is limiting, for example retro-
viruses, it might be desirable to transfer only the gene of
interest and not the remaining 2 kb of the univector. To
accomplish this, we devised the POT strategy. POT uses
a second recombination event catalyzed by the R recombi-
nase[13] that allows the resolution of a UPS-generated
two-plasmid fusion as shown in Figure 3a. We placed an R
recombination site,RS, after the cloning site in pUNI20
such that any gene inserted into pUNI20 would be
flanked byloxP andRS. Recipient vectors must also
containloxP andRS elements in the same order. POT can
be carried out in two sequential steps or in one step. For
the two-step method, the initial fusion event is achieved
by UPS. The second step can be catalyzed either in vitro
by incubation with purified R recombinase[13], or in vivo
by transformation into a strain (BUN15) expressing the R
recombinase under the control of thelac promoter on a
temperature-sensitive replication plasmid (pML66) which
is lost when cells are plated at 42°C. R-mediated recombi-
nation can achieve 30% efficiency in vitro and 15% effi-
ciency in vivo (data not shown).

POT can be achieved efficiently in a single step with a
selection through the use of a counterselectable marker
that is placed betweenloxP andRS on pHOST. For this
purpose we have used theΦX E gene which is toxic
when expressed inE. coli unless the host cell lacks the
slyD gene encoding a cis-trans peptidyl-prolyl isomerase
[14]. A two-hybrid recipient vectorpAS2-E was con-
structed frompAS2[15] that contains (from 5′ to 3′)loxP,
tac-E, andRS. After a UPS reaction between
pUNI20-SKP1 and pAS2-E, the reaction mixture was
transformed directly into BUN15 cells (pir− slyD+) in
the presence of IPTG to induce E expression selecting for
ampicillin resistance. Due to the toxicity ofE, the only
ApR transformants surviving would be those containing
the recombinants that have undergone POT. The toxic
ΦX E gene along with the univector backbone will be
eliminated by the R-dependent site-specific recombi-
nation, resulting in the replacement ofE on pAS2-E by
SKP1. As shown in Figure 3b, 100% (20 out of 20) of ApR
transformants contained the desired recombinant products as determined by restriction analysis with PvuII.

Generation of 3′ gene fusions using homologous recombination in E. coli

Although UPS greatly facilitates the generation of fusion proteins at the amino terminus of the protein of interest, it is often necessary to modify proteins at the carboxyl terminus. To facilitate this class of modification, we have taken advantage of the endogenous homologous recombination system of E. coli. It has been previously shown that E. coli strains mutant for the recombination genes recBC and sbc [16] or strains mutant for recD [17] can take up linear DNA and recombine it into the E. coli chromosome or resident plasmids. We therefore generated BUN10, a recBCsbcAhsdR strain expressing pir-116. The hsdR mutation is needed to prevent restriction of unmethylated PCR-amplified DNA by the endogenous E. coli restriction enzyme encoded by hsdR. We tested this by tagging the SKP1 gene in pUNI at its carboxyl terminus with the Myc 3 tag. An ApR pUNI derivative containing a MYC3 epitope tag followed by a stop codon, pML74, was used as template DNA for PCR amplification. Primer A (71 nt) contains at its 5′ end 51 nt which correspond to the last 51 nt of the SKP1 ORF (excluding the stop codon) and at its 3′ end the first 20 nt of the DNA encoding the Myc3 tag in-frame with the SKP1.
ORF. Primer B (22 nt) recognizes a site on pML74 common to all pUNI vectors that begins 367 bp from the MYC3 region. Thus, PCR-amplified DNA will contain a MYC3 tag flanked by 51 bp homology to the SKP1 gene and 367 bp homology to the univector. This PCR fragment, together with BamHI–SacI-linearized pUNI20-SKP1 DNA, was co-transformed into BUN10 cells and KnR transformants were selected. Homologous recombination at the carboxyl terminus of the SKP1 gene to generate a SKP1–MYC3 fusion occurred in 95% of KnR transformants (data not shown). This pUNI-SKP1–MYC3 construct was fused to pHY326 to generate a 2μ URA3 GAL-SKP1–MYC3 expression construct. Expression of the fusion protein could be detected in S. cerevisiae (Figure 3d). This demonstrates that homologous recombination in E. coli can be used to alter the sequence of genes at the 3’ end using PCR-derived material.

**Methods that facilitate gene transfer into the univector and other plasmids**

Subcloning DNA into vectors is limited by the restriction sites available. When cloning blunt-ended DNA molecules such as those generated by thermostable polymerases, it is desirable to have a way of identifying recombinant molecules. This is of significant interest to us because the initial cloning of genes into pUNI will often use PCR-amplified material. To facilitate this process, we have developed a method for directional cloning into pUNI derivatives that relies upon the generation of a lac operator site upon ligation. Plasmid and phage carrying the binding site for the lac repressor, lacO, could induce the expression of the endogenous lacZ gene by titrating out a limiting number of repressor proteins [18–20] (Figure 4a). We took advantage of this observation by placing the 3’ half of a modified lacO site in pUNI30 (Figure 4b). This lacO derivative is a symmetrical 20 bp site with an Eco47III restriction site at the center. PCR primers were made to amplify the bla gene. To the 5’ end of the 3’ primer, an extra 10 bp was added that corresponds to the 5’ half of the symmetrical lacO site. The PCR-amplified DNA was ligated to Eco47III-cleaved pUNI30, transformed into BUN10 (pir-116 lac+), and selected on plates containing kanamycin and X-gal. Plasmids containing bla in the correct orientation were identified by their dark blue color (Figure 4c): 11 out of 12 dark blue colonies contained full-length bla in the correct orientation (data not shown). This method works best when the PCR primers used are phosphorylated. If Taq polymerase is used, it is necessary to briefly treat the material with T4 DNA polymerase and dNTPs to remove 3’ overhangs.

**Library transfer using UPS**

One of the most significant advances made possible by the UPS method is the generation of libraries in the univector that can then be transferred into other specialized vectors, essentially turning one library into many libraries. We tested this possibility by making a randomly sheared S. cerevisiae genomic library in pUNI10 using the XhoI-adaptor strategy [21]. This library had 5 × 10⁵ recombinants with 80% of inserts ranging from 3 to 8 kb. This library was fused using UPS to a 2μ URA3 plasmid pRS426-lox and 2 × 10⁵ KnR transformants were recovered. We demonstrated that the converted library maintained its representation by examining the presence of 42 different ORFs by PCR. Both the pre-UPS and post-UPS libraries contained all 42 ORFs (data not shown). This
UPS-derived 2µ yeast genomic library was used to complement a temperature-sensitive mutation in the F-box protein Cdc4, cdc4-1 [22], at the non-permissive temperature (34°C). Three classes of suppressors were recovered, CDC4, SKP1 and the mitotic cyclin CLB3 (data not shown). SKP1 and CLB4, a cyclin closely related to CLB3, had been previously shown to suppress cdc4-1 mutants when overexpressed from the GAL1 promoter [11,22]. These experiments demonstrate the feasibility of library transfer using UPS. In cases where a cDNA expression library is created, such as for the two-hybrid system, once positive clones have been isolated they can rapidly be converted back into simple univector clones by Cre recombinase. These univector clones can then be fused with any pHOST by UPS to generate expression constructs for future analytical needs.

Discussion

Unlike the conventional ‘cut-and-paste’ strategy of restriction-enzyme-based methods, recombinant DNA assembled by UPS is achieved by plasmid fusion through site-specific recombination. UPS can be used to fuse a coding region of interest either with a specific promoter to gain novel transcriptional regulation, or with another coding sequence to produce a fusion protein with new properties. UPS eliminates the use of restriction enzymes and DNA ligase: instead, these functions are both carried out simultaneously by a single enzyme, Cre. This relieves the constraints on cloning vectors with respect to DNA sequence and size because the UPS reaction is independent of vector size or sequence. Furthermore, the time-consuming processes inherent in conventional cloning, such as the identification of a suitable vector, designing a cloning strategy, restriction endonuclease digestion, agarose gel electrophoresis, isolation of DNA fragments, and the ligation reaction, is shortened to a 20 minute UPS reaction. Due to the uniformity and simplicity of UPS, dozens of constructs can be made simultaneously by simply using different recipient vectors. In addition, unlike restriction enzymes and DNA ligases, GST–Cre can be made inexpensively in large quantities. These features will save investigators significant amounts of time and expense.

The univector has a number of features that make it especially useful for the UPS method. It is small, adding only a minimal amount of DNA to a construct, and brings in its own transcriptional termination signals, which simplifies the recipient vector construction. Its conditional origin of replication can exist at two different copy numbers depending on the pir allele present in the bacterial strain used for propagation [9]. The wild-type pir+ allele confers a low copy number, 15 copies per cell, while the pir-116 allele confers a high copy number, 250 copies per cell. This property may be useful when potentially toxic genes are manipulated. Furthermore, the only promoter present in the univector is that driving the neo gene, which is transcribed in the opposite direction from cDNAs cloned into the univector. This lessens the chances that a toxic gene will be expressed.

While at present we have generated approximately 40 pHOST vectors (Q.L., S.J.E. and J.W. Harper, unpublished), we are in the process of generating prototype recipient vectors for all general expression needs. We have successfully tested bacterial GST, yeast GAL1 and mammalian CMV expression vectors and many baculovirus expression vectors. In each case, expression was at or near the levels achieved by conventional cloning methods. The generation of pHOST vectors is simple, requiring only the insertion of an oligonucleotide linker containing a loxP or loxH site into pre-existing vectors.

We have developed additional methods to facilitate gene manipulation by UPS. POT employs both the Cre–lox and R–RS site-specific recombination to precisely transfer an ORF from pUNI to pHOST. We have devised a simple directional PCR cloning method to facilitate the introduction of genes into the univector based on the reconstitution of a lacO site upon ligation. Finally, we have solved the problem of generating 3′ end gene fusions using UPS by taking advantage of endogenous homologous recombination in E. coli. These methods make gene manipulation using UPS a facile process.

Future uses of the UPS

The high efficiency of the in vitro UPS reaction coupled with the highly efficient electroporation method of bacterial transformation makes possible the conversion of whole cDNA libraries constructed in the univector into expression libraries in a different vector without loss of representation, as we demonstrated with a yeast genomic library. Thus, a single library could be converted into a number of different expression libraries and will no longer need to be remade from scratch when needed in a different context. Clones isolated from these libraries will be easily converted back into simple univector plasmids compatible with other pHOST vectors for future analysis. UPS is compatible with the λYES series of lambda cloning vectors which use Cre–lox recombination to convert phage libraries into plasmid libraries [21] and which are capable of making extremely large cDNA libraries (>10⁶ recombinants per 100 ng of cDNA) and, unlike plasmid libraries, can be propagated with minimal loss of representation.

One promising future application of the UPS method is the manipulation of whole genome sets of coding regions. For organisms for which the genomes have been sequenced, a complete set of identified ORFs can be constructed in the univector to make a ‘Unigene’ array ready for transfer into any expression system. Furthermore, the simplicity and uniformity of the UPS reaction makes it...
readily amenable to automation for systematic conversion of arrayed clones. This will greatly expedite the functional characterization of whole genomes and help further the progression of genome projects into proteome projects.

**Conclusions**

In this study, we have described a novel and broadly applicable approach to the generation of recombinant DNA. This new series of recombinase-based cloning technologies overcomes many of the defects associated with the currently available conventional cloning methods. Together, these methods constitute a comprehensive strategy for the rapid and systematic generation and manipulation of recombinant DNA molecules without the use of restriction enzymes. Furthermore, these recombinase-based technologies open new avenues for the systematic manipulation and processing of large gene sets in parallel, a feature that is essential for future functional genomic research.

**Materials and methods**

**Media, enzymes and chemicals**

For drug selections, LB plates or liquid media were supplemented with either kanamycin (50 µg/ml), ampicillin (100 µg/ml) or spectinomycin (50 µg/ml). When necessary, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.3 mM and X-gal was used at 80 µg/ml. Yeast growth media and plates were made as described [23]. Restriction endonucleases, large (Klenow) fragment of *E. coli* DNA polymerase I, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase were purchased from New England Biolabs. Drugs were purchased from Sigma if not specified.

**Bacterial and yeast strains**

*E. coli* BW23474 ([Jac+169, ropS(am), robA1, creC510, hasdR514, uidA(ΔMlu):parR116, endA, recA1] and BW23473 [Jac+169, robA1, creC510, hasdR514, uidA(ΔMlu):pirR116, endA, recA1] [24]) were a gift of B. Wanner. The full life cycle of all strains for propagation of all univector plasmids. CX1 (stryD) was used for propagation of E expressing plasmids [14]. JM107 or DH5α was used as recipient host for US reactions. BUN10 [hisG4 thr-1 leuB6 t lacY1 kdgK51 gpt-proA]62 rpsL31 ttx33 supE44 recB21 recC22 sibc23 hasd:cat-pir116(CmR)] was used for homologous recombination experiments. BUN13, which has cre under the control of the lac promoter, is JM107 lysogenized with λc (aadA lac-re). BUN15 is XL1 blue containing pML66 (lac- lac- SpR) and was used for the in vivo recombination assays. B. coli BL21 was used for bacterial expression studies. *S. cerevisiae* Y800 [25] was used for yeast transformation studies and Hi5 cells were used for baculovirus expression. Y543 (as Y800 but cdcd4-1) was used for cdcd4 suppression [22].

**Construction of BUN13**

The pS356 plasmid (S.J.E., unpublished) contains a cassette consisting of the Tn5 neo gene, the lac promoter and a polylinker sequence surrounded by λ DNA sequence. The pQL114 plasmid was constructed in two steps. First, the BamHI– HindIII (blunt-ended) fragment containing aadA (SpR) from pDPT720 [26] was subcloned into BamHI– Sph I (blunt-ended) pSE356 to create pQL102, replacing neo with aadA. Then a Pcl-PCR fragment was introduced into pQL102, placing cre under lac control adjacent to aadA and flanked by λ DNA. The λc phage [21] was amplified on JM107 containing pQL114 and the resulting phage lysate containing the desired recombinant λc phage was used to infect JM107. SpR KnR lysogens were selected and a strain positive for cre expression and the ability to perform UPS was named BUN13.

**Plasmid construction**

The cre gene was amplified by PCR to place an Ncol site at the first ATG using primers 5′-CCATGGGCAATTTACGTACGCTAC-3′ and 5′-CCCCCGGTATACTGCATTTCTGACCA-3′. The PCR product was cloned into pCR™ and subcloned into pGEX-2Tkcs (W.J. Harper, unpublished) as an Ncol– EcoRI fragment to create pQL123. The pHOST plasmid pQL103 was made by deleting one of two loxP sites from pSE1086 (S.J.E., unpublished) between NotI and SalI. The 590 bp Ncol–BamHI SKP1 ORF was subcloned from pCB149 [11] into Ncol–BamHI-cleaved pUNI10 to create pQL130 (pUNI10-SKP1). The pUNI-RAD53 (pQL135) clone was constructed by subcloning the Hα–RAD53 gene as an Ncol–NotI (blunt-ended) fragment from pQL49 into Ncol–BamHI (blunt-ended) pUNI10. The MYC–RNR4 gene was subcloned from pMH176 [27] into pUNI10 to create pQL248, or into pBAD104 (B. Denaney, unpublished) to create the control GAL:MYC–RNR4 plasmid pQL222. The pQL138 and pQL193 plasmids were derived from pBAD104 by insertion of oligonucleotide linkers containing loxP (5′-TCGAGACGTAT- AACCTGTATAGCATACTATTAGGAATGTCG-3′ and 5′-GGCGATACTTGTATAATGTATGAGCATGCATGACC-3′) or loxH (5′-CATGGCTATACCTGATGAGCTGATTACGAGTATG-3′) and 5′-GATCCATACCTGTATAGTATGAGCTGATTACGAGG-3′, respectively. The pMH13, pQL124–RNR4 constructs were made by UPS using pQL248 with pQL138 or pQL193. The construction details of pHB2-GST, pHY314-MYCp, pYH326, pHI100-GST, pHI100-MYCp, pHI100-HAp and pHM200-HAp are available upon request. The pQL269 plasmid (lac–cre, SpR and onR1) was constructed by ligating the EcoRI– PvuII fragment from pQL114, containing aadA (conferring spectinomycin resistance) and the lac–cre chimeric gene, to a BglII(blunt)– EcoRI fragment containing the temperature-sensitive replication origin from pM175 [28]. The pML66 was constructed by ligating the EcoRI– SalI (blunt-ended) fragment containing the lac promoter driving the R recombinase from pNN115 [13] to EcoRI– PstI (blunt-ended) cleaved pQL269. The pML73 plasmid, the pUNI20-SKP1 clone used for POT, contains the full-length SKP1 ORF plus additional 800 bp 3′ flanking genomic sequence. The pAS2-E plasmid (pML71) was constructed by first inserting a loxP site between the Ncol and SalI sites of pAS2 [15] followed by a three-way ligation using a Xhol–SpeI fragment containing tac-E, a SpeI– PstI synthetic RS oligonucleotide linker, and SalI– PstI linearized pAS2-lox. The pML74 plasmid was made for first replacing the neo gene on pUNI20 with the bα gene from pUC19 and subsequently inserting the MYC3 tag that was PCR amplified from pJBN48 (J. Bechant, unpublished; details upon request). The PCR primers used to amplify MYC3 from pML74 for the 3′ tagging of SKP1 were primer A (MIZ179) 5′-CCGCGAGAGGGCGCTTGGACACCCACGCTCAG-3′ and primer B (MIZLE1) 5′-GGTATATGCTCTTCCCTTCCAG-3′.

**Expression and purification of the GST–Cre fusion protein**

*E.coli* BL21 cells containing pQL123 were grown at 37°C in LB containing ampicillin to an OD600 of 0.4. IPTG was then added and the culture was incubated at 25°C overnight. The GST–Cre fusion protein was purified using glutathione–Sepharose beads (Pharmacia) according to the manufacturer’s instructions. Purified GST–Cre can be stored at −80°C, −20°C or 4°C for several months without significant loss of activity.

*In vitro UPS and POT assays*

For UPS assays shown in Figure 1c, 0.4 µg DNA of each of pUNI10 and pQL103 were mixed with purified GST–Cre in a total volume of 20 µl in 1 x buffer S (50 mM Tris HCl (pH 7.5); 10 mM MgCl2, 30 mM NaCl, 0.1 mg/ml BSA). The reaction mixtures were assembled on ice and incubated at 37°C for 20 min following by incubation at 65°C for 5 min to inactivate GST–Cre. Typically half of the reaction mixture was transfomed into DH5α or JM107 cells, and the transformants were
selected on LB plates containing either ampicillin or kanamycin. For
POT assays, BUN15 cells were grown overnight in LB containing
spectinomycin, diluted to 1 in 100 in fresh LB containing spectino-
mycin and IPTG and grown to OD_{600} of 0.5 at 30°C, and electrocom-
petent cells were prepared as recommended (BioRad). In each
transformation, 40µl competent cells were used, and after the electro-
poration cells were incubated in LB containing IPTG for 1–4 h at 42°C
before being plated on LB/Amp/IPTG (1 mM) at 42°C.

In vivo UPS assays
BUN13 electrocompetent cells were prepared in LB containing 2%
glucose and 1 ml LB (0.5% glucose) for 1 h at 42°C before being plated
onto LB/Amp or LB/Kan plates containing 0.5% glucose at 42°C.

β-galactosidase assays
Yeast cells expressing GAL1:lacZ reporter constructs were grown at
30°C to mid-log phase (OD_{600} = 0.5–0.6) in SC-Ura media containing
2% raffinose; galactose was added to 2% final concentration and cells
were cultured in SC-Ura media containing either ampicillin or kanamycin. BUN14
cells were grown in LB media to OD_{600} of 0.5 at 42°C and competent cells were made
as described for BUN15. After electroporation, cells were incubated with
1 ml LB (0.5% glucose) for 1 h at 42°C before being plated onto
LB/Amp or LB/Kan plates containing 0.5% glucose at 42°C.

Supplementary material
A figure showing the PCR analysis of pre-UPS and post-UPS libraries is published with this paper on the internet.

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References
1. Smith HO, Wilcox KW: A restriction enzyme from Hemophilus
influenzae. I. Purification and general properties. J Mol Biol 1970,
51:379-381.
2. Danna K, Nathans D: Specific cleavage of simian virus 40 DNA by
restriction endonuclease of Hemophilus influenzae. Proc Natl
Acad Sci USA 1971, 68:2913-2917.
3. Cohen SN, Chang AC, Boyer HW, Helling RB: Construction of
biologically functional bacterial plasmids in vitro. Proc Natl Acad
4. Backman K, Ptashne M: Maximizing gene expression on a plasmid
5. Sternberg N, Hamilton D, Austin S, Yarmolinsky M, Hoess R: Site-
specific recombination and its role in the life cycle of P1. Cold
6. Abremski K, Hoess R, Sternberg N: Studies on the properties of P1
site-specific recombination: evidence for topologically unlinked
7. Sauvageau G: Functional expression of the cre-lox site-specific
recombination system in the yeast Saccharomyces cerevisiae. Mol
8. Oriban PC, Chu D, Marth JD: Tissue- and site-specific DNA
recombination in transgenic mice. Proc Natl Acad Sci USA 1992,
89:6861-6865.
allele replacement to construct new Escherichia coli hosts for
maintenance of R6K gamma origin plasmids at different copy
expressed in Escherichia coli as fusions with glutathione
connects cell cycle regulators to the ubiquitin proteolysis
machinery through a novel motif, the F-box. Cell 1996,
86:263-274.
12. Kozak M: Circumstances and mechanisms of inhibition of
translation by secondary structure in eucaryotic mRNAs. Mol Cell
13. Araki H, Nakanishi N, Evans ER, Matsuzaki H, Jayaram M, Oshima Y:
Site-specific recombinase, R, encoded by yeast plasmid pSR1.
14. Maratea D, Young K, Young R: Deletion and fusion analysis of the
15. Durfee T, Beckerer K, Chen PL, Yeh SH, Yang Y, Kilburn AE, et al.: The
directed insertion and deletion mutagenesis with cloned
17. Russell CB, Thaler DS, Dahlquist FW: Chromosomal transformation
of Escherichia coli recD strains with linearized plasmids. J
synthetic operator DNA is functional in vivo. Nature 1976,
263:744-748.
20. Heynekeler NL, Shine J, Goodman HM, Boyer HW, Rosenberg J,
Dickerson RE, et al.: Synthetic lac operator DNA is functional in
21. Elledge SJ, Mulligan JT, Ramer SW, Spottswood M, Davis RW:
Lambda YES: a multifunctional cDNA expression vector for the
isolation of genes by complementation of yeast and
13:6087-6098.
23. Rose ND, Winston F, Hieter P: Laboratory Course Manual for
Spring Harbor Laboratory Press; 1990.
24. Metcalf WW, Jiang W, Daniels LL, Kim S, Halldman A, Wanner BL:
Conditionally replicative and conjugal plasmids carrying lacZa
for cloning, mutagenesis, and allele replacement in bacteria
transcription of the DNA damage inducible gene RNR3 in
DNA segments containing the replication and incompatibility
regions of a miniplasmid derived from a copy number mutant of
27. Huang M, Elledge SJ: Identification of RNR4, encoding a second
essential small subunit of ribonucleotide reductase in
28. Hasan N, Koob M, Szybalski W: Escherichia coli genome targeting,
I. Cre-lox-mediated in vitro generation of ori- plasmids and their
in vivo chromosomal integration and retrieval. Gene 1994, 150:51-56. Because Current Biology operates a ‘Continuous Publication System’ for Research Papers, this paper has been published on the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cub for further information, see the explanation on the contents page.
Examination of the library representation of pre-UPS and post-UPS libraries by PCR: 42 pairs of primers were used to amplify 42 different ORFs (in some cases only portions of ORFs were amplified) from both the pre-UPS and post-UPS libraries. For each ORF, the PCR products for the two libraries were loaded in adjacent lanes.