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

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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.

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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 (His_6) 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

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 multifaceted 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 is 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 *Nde*I and *Nco*I 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, Kn^R) 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).

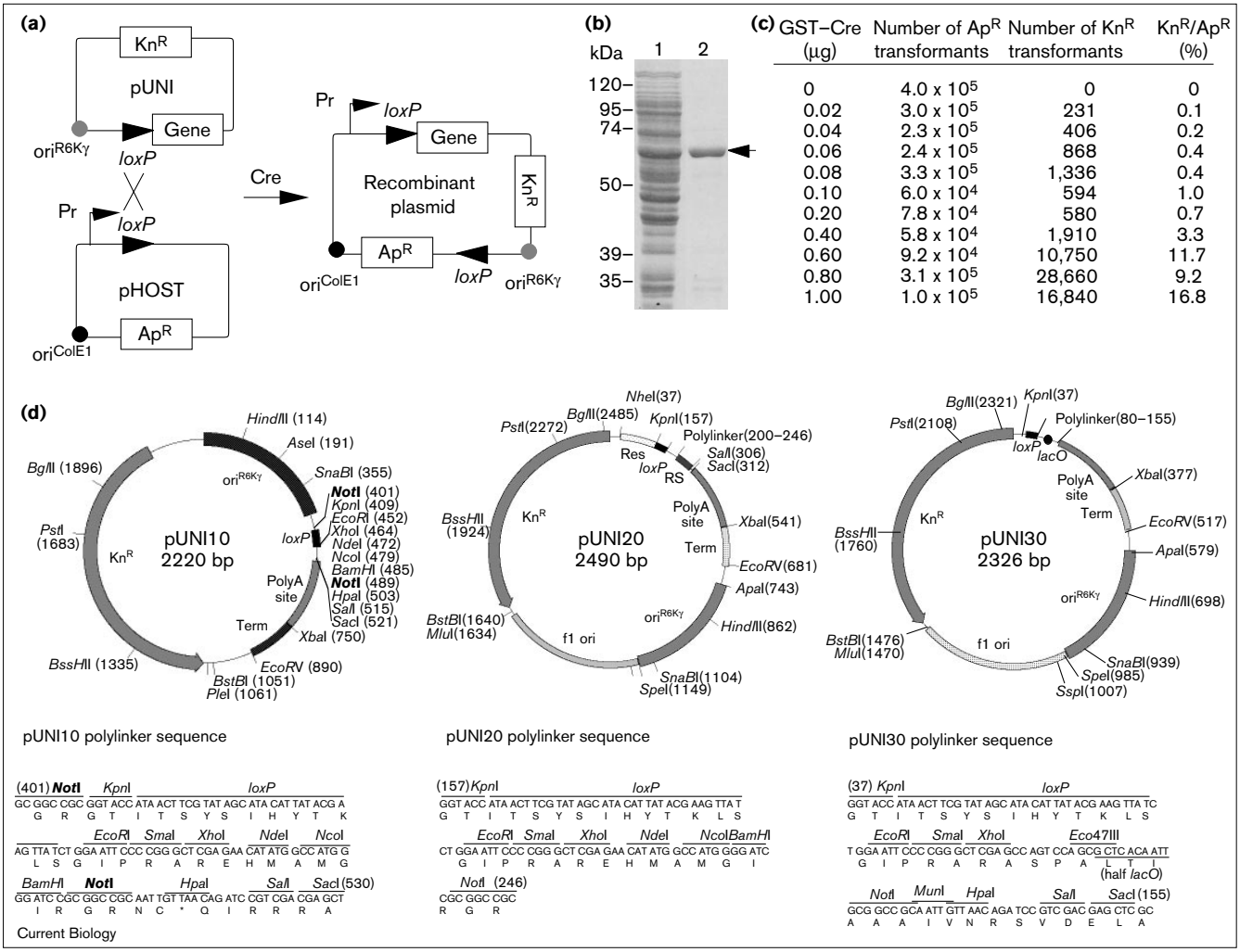
The pHOST vector features include the ColE1 origin of replication, the *bla* gene (which encodes ampicillin resistance, Ap^R) for propagation and selection in bacteria, a *loxP* site for plasmid fusions and a specific promoter residing upstream of, and immediately adjacent to *loxP*. Host vectors may 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 a 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 (ori^{ColE1}) 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

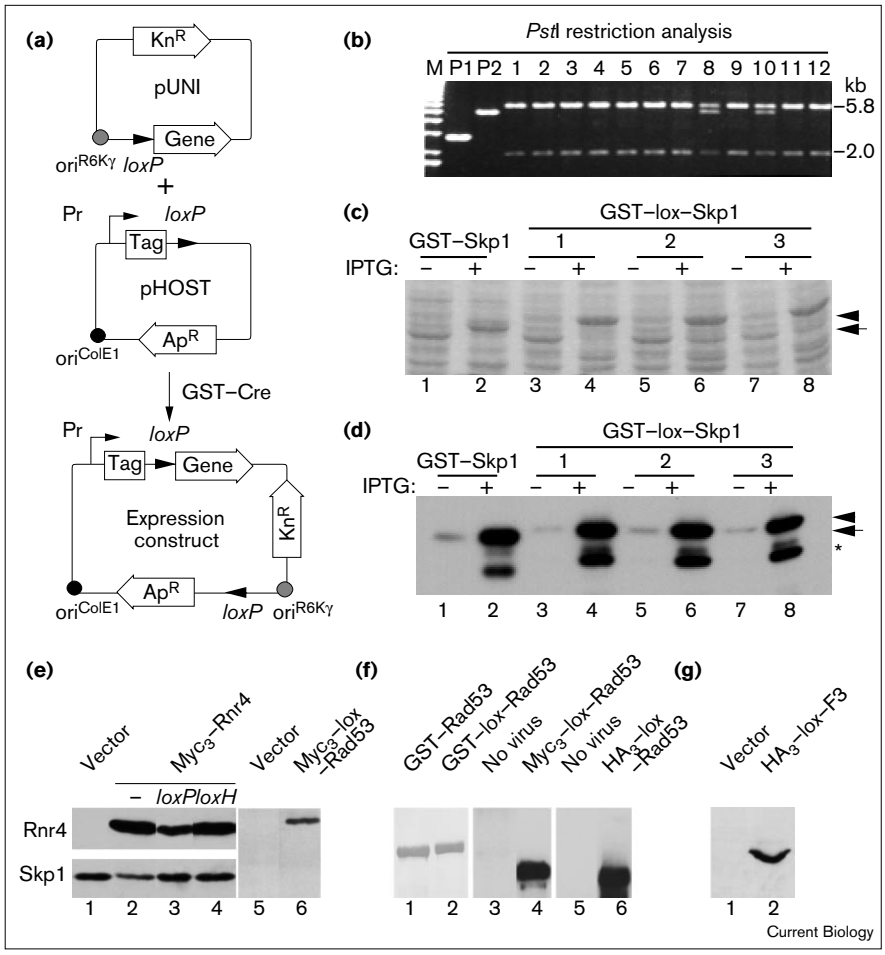
Figure 1



(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 beads. **(c)** Recombinase activity of GST–Cre. UPS reactions were carried out with different amounts of GST–Cre as described in Materials and methods. The efficiency of the UPS reactions is shown as the ratio of total Kn^R transformants to total Ap^R transformants. **(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 ORF 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 Kn^R 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 Kn^R 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.

Figure 2



Expression of UPS-derived constructs in multiple systems. **(a)** A schematic representation of the generation of epitope-tagged expression constructs by UPS. **(b)** A *Pst*I restriction analysis of the UPS-derived GST–lox–Skp1 recombinant plasmids. Plasmid DNA from 12 independent Kn^R transformants was digested with *Pst*I 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 (pQL130; lane P1), and pHB2-GST (lane P2), each contain a single *Pst*I 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 (c) stained with Coomassie blue or (d) 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. **(e)** Expression 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:MYC$_3$–RNR4* (pQL222) lacking a *lox* site (lane 2), UPS-derived *GAL:MYC$_3$–RNR4* constructs containing *loxP* (lane 3) or *loxH* (lane 4); vector alone (pHY314-MYC$_3$; lane 5), or *GAL:MYC$_3$-RAD53* (pUNI-RAD53::pHY314-MYC$_3$) 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. **(f)** Expression of UPS-derived baculovirus constructs in insect cells. The pUNI-RAD53 plasmid was fused by UPS to pHI100-GST, pHI100-MYC$_3$ or pHI100-HA$_3$ to generate GST–Rad53, Myc$_3$–Rad53 and HA$_3$–Rad53, respectively. These recombinant plasmids were used to make baculovirus. Total cell extracts are prepared from 2×10^6 cells infected with baculovirus derived from GST–Rad53 constructs made by conventional cloning (lane 1) or UPS (lane 2), no virus (lane 3), Myc$_3$–Rad53 (lane 4), no virus (lane 5) and HA$_3$–Rad53 (lane 6). Gels were Coomassie stained (lanes 1,2) or immunoblotted with anti-Myc (lanes 3,4) or anti-HA antibodies (lanes 5,6). **(g)** Expression of UPS-derived constructs in mammalian cells. Hela cells were transfected by the calcium phosphate method with the CMV expression vectors pHM200-HA$_3$ or pHM200-HA$_3$–F3, expressing an HA-tagged F-box protein. Whole cell lysates were subjected to immunoblotting with anti-HA antibodies.

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**TA**AC**TTC**GT**AT**T; 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, various *loxP* mutants with decreased stem-loop stability tended to result in better expression of β-galactosidase. One mutant, *loxH* (AT**T**AC**C**TC**A**TATAGCATACATTATACGAAGTTAT; mutations in bold), failed to display inhibitory effects when compared with *loxS* (data not shown). The effects of *loxH* were further tested by using it to place the gene encoding the ribonucleotide reductase Rnr4 protein tagged with three copies of the Myc epitope, *MYC_3–RNR4,* under the control of the *GAL1* promoter; *loxH* showed improved translational ability relative to *loxP* (Figure 2e, compare lanes 2–4). It should be noted that the control lacking a *lox* site in Figure 2e has a different spacing and is not completely isogenic for the purposes of comparing translational efficiency of *loxH*. When paired with *loxP*, *lox*H also retained 25% of the wild-type recombinational efficiency (data not shown) which is well within the useful range for UPS-mediated plasmid construction. We recommend that *loxH* be used in recipient vectors intended for transcriptional fusions to maximize expression, whereas *loxP* 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, Myc_3-tagged versions of Rnr4 and the checkpoint protein Rad53 have been expressed in *S. cerevisiae* (Figure 2e). Furthermore, many baculovirus expression constructs have been made by UPS and tested (our unpublished observations) and shown here are GST–Rad53, Myc_3–Rad53, and HA_3–Rad53 (Rad53 tagged at the amino terminus with three copies of the hemagglutinin (HA) tag; Figure 2f). The UPS-derived construct encoding GST–Rad53 expressed it 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 HA_3-tagged F-box protein under the control of the cytomegalovirus (CMV) promoter in Hela cells (Figure 2g). Over 200 UPS-derived constructs show expression success rates indistinguishable from those of conventional cloning methods for all genes and all organisms tested thus far.

In vivo UPS

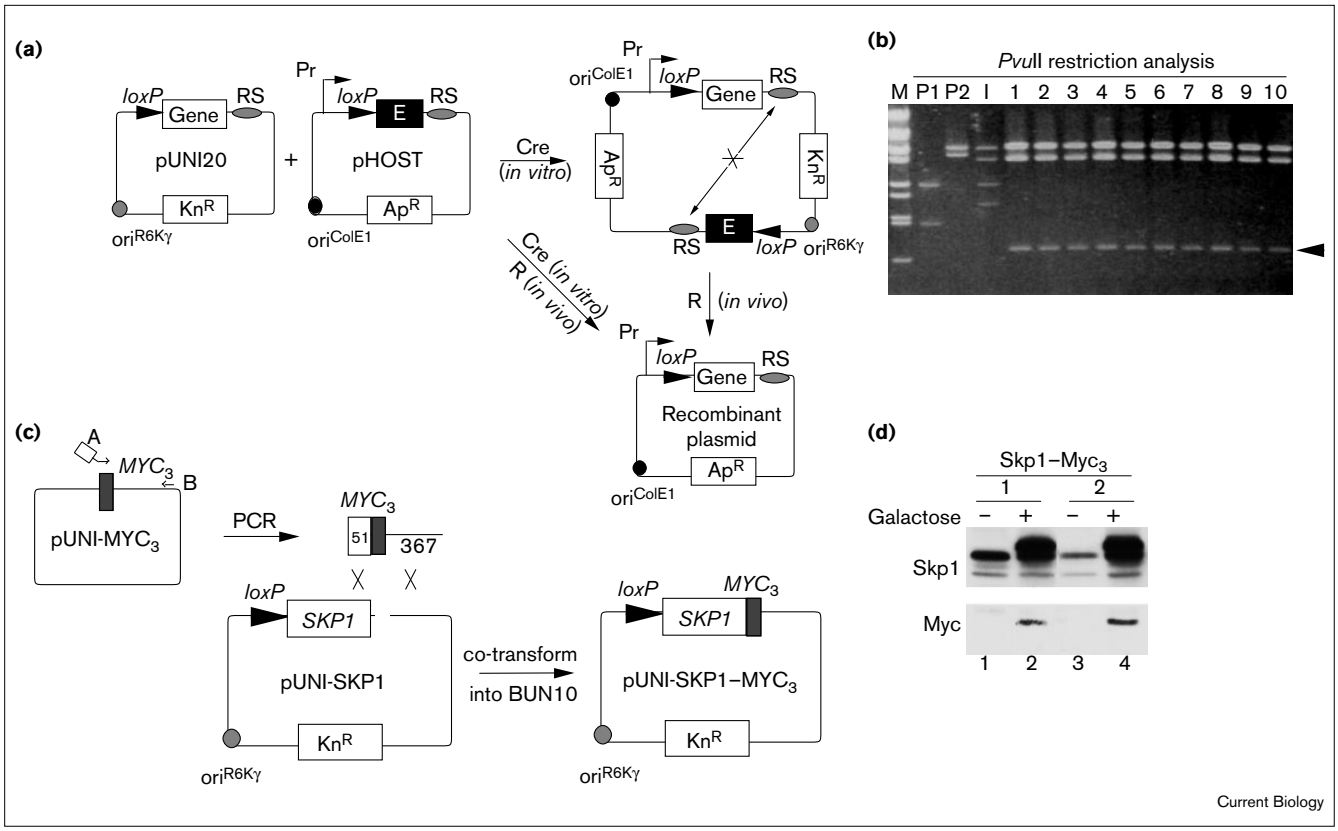
Cre–*loxP*-mediated plasmid fusion can also occur *in vivo*. Ideally, it would be desirable to have Cre present only transiently to catalyze the initial fusion event, then absent to allow the stable propagation of the recombinant products. We therefore explored UPS *in vivo* in the *E. coli* strain BUN13 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 pSC101. Experiments using BUN13 and co-transformation 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-tranformation of pUNI10 and pQL103 into these cells followed by kanamycin selection at 42°C revealed that 25% of transformants contained the desired single pUNI10–pQL103 co-integrant. 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 retroviruses, 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 recombinase [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 by *loxP* and *RS*. Recipient vectors must also contain *loxP* and *RS* 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 the *tac* promoter on a temperature-sensitive replication plasmid (pML66) which is lost when cells are plated at 42°C. R-mediated recombination can achieve 30% efficiency *in vitro* and 15% efficiency *in vivo* (data not shown).

POT can be achieved efficiently in a single step with a seletion through the use of a counterselectable marker that is placed between *loxP* and *RS* on pHOST. For this purpose we have used the ΦX *E* gene which is toxic when expressed in *E. coli* unless the host cell lacks the *slyD* gene encoding a *cis-trans* peptidyl-prolyl isomerase [14]. A two-hybrid recipient vector pAS2-E was constructed from pAS2 [15] that contains (from 5′ to 3′) *loxP*, *tac-E* and *RS*. 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 of *E*, the only Ap^R 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 recombination, resulting in the replacement of *E* on pAS2-E by *SKP1*. As shown in Figure 3b, 100% (20 out of 20) of Ap^R

Figure 3



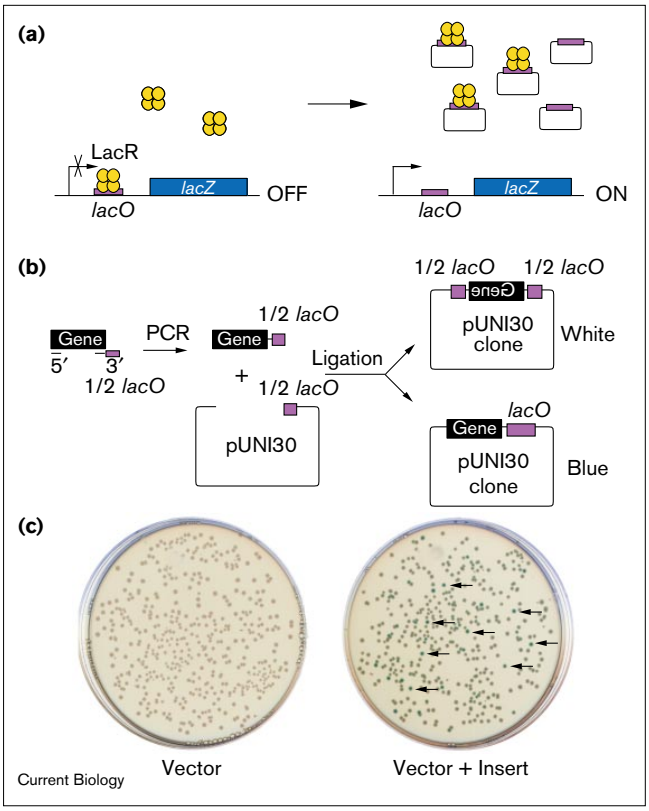
Precise ORF transfer (POT) and carboxy-terminal epitope-tagging of proteins using homologous recombination in *E. coli*. **(a)** A schematic representation of POT. The initial fusion event of a two-step POT reaction is catalyzed by UPS followed by a second resolution reaction catalyzed by R-mediated recombination that eliminates both the toxic ΦX *E* gene and the univector backbone. The second step can be performed *in vitro* with purified R recombinase or *in vivo* by transformation into BUN15 which conditionally expresses R. POT can also be achieved in a single step by directly transforming the UPS reaction mixture into BUN15 (see text). **(b)** A *Pvu*II restriction analysis of the recombinant plasmids generated by one-step POT between pUNI20-SKP1 (pML73) and pAS2-E. P1 and P2 refer to pUNI20-SKP1 and pAS2-E, respectively. I represents the intermediate generated by plasmid fusion. The analysis of 10 Ap^R transformants is shown in lanes 1–10 and a new 800 bp band is indicative of POT and is indicated by an arrowhead. **(c)** A diagram showing 3′ end gene fusion via homologous recombination in *E. coli*. Primers A and B (see Materials and methods) are used to amplify the MYC_3 tag from pML74, generating a PCR fragment containing MYC_3 flanked by 51 bp homology to the 3′ end of the *SKP1* ORF and 367 bp homology to the univector (see text). The blunt-ended PCR product was co-transformed into BUN10 with pUNI20-SKP1 (pML73) DNA linearized 3′ to *SKP1*. Homologous recombination between two linear DNA fragments result in a circular plasmid containing *SKP1* fused to MYC_3 at its carboxyl terminus. **(d)** Expression of Skp1–Myc_3 generated by homologous recombination. Two pUNI-SKP1–MYC_3 plasmids generated in (c) were fused to pHY326 by UPS to generate *GAL:SKP1–*MYC_3 plasmids and proteins expressed from these in the yeast strain Y80 in the presence of glucose or galactose were detected by immunoblotting with anti-Skp1 and anti-Myc antibodies.

transformants contained the desired recombinant products as determined by restriction analysis with *Pvu*II.

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 Ap^R pUNI derivative containing a MYC_3 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 Myc_3 tag in-frame with the *SKP1*

Figure 4



Directional cloning of PCR fragments by reconstituting a functional *lac* operator. **(a)** A diagram illustrating induction of the endogenous *lacZ* gene by titration of the *lac* repressor by a high copy number plasmid containing *lacO* sites. Normally, the endogenous *lacZ* gene is repressed by *lac* repressors bound to its *lacO* sites (left). When *lacO* sites are present on a high copy number plasmid, however, the *lac* repressor molecules are bound by plasmids (right), thereby derepressing the transcription of *lacZ*. **(b)** A representation of directional PCR cloning by *lacO* reconstitution. Primers A (5′) and B (3′) are used to amplify the gene of interest. The 5′ end of primer B contains a half *lacO* site which subsequently becomes the 3′ end of the PCR fragment as shown. After ligating the PCR fragment into *Eco*47III-linearized pUNI30 that contains the other half of *lacO*, an intact *lacO* site is reconstituted and, in *lac*$^+$ cells, results in induction of endogenous β-galactosidase and blue color in the presence of X-gal. **(c)** Photos of colonies from a ligation as in (b) transformed into BUN10 cells (*lac*$^+$) and plated on LB plates containing kanamycin and X-gal. The reconstituted *lacO* sites result in blue colonies (arrows) due to induction of the endogenous *lacZ*. Reclosure of the vector without insert or insertion of the PCR fragment in the incorrect orientation results in white or pale blue colonies.

ORF. Primer B (22 nt) recognizes a site on pML74 common to all pUNI vectors that begins 367 bp from the MYC_3 region. Thus, PCR-amplified DNA will contain a MYC_3 tag flanked by 51 bp homology to the *SKP1* gene and 367 bp homology to the univector. This PCR fragment, together with *Bam*HI–*Sac*I-linearized pUNI20-SKP1 DNA, was co-transformed into BUN10 cells and Kn^R transformants were selected. Homologous recombination at the carboxyl terminus of the *SKP1* gene to generate a *SKP1–MYC*$_3$ fusion occurred in 95% of Kn^R transformants (data not shown). This pUNI-SKP1–MYC$_3$ construct was fused to pHY326 to generate a 2μ *URA3 GAL:SKP1–MYC*$_3$ 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 a *Eco*47III 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 *Eco*47III-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 *Xho*I-adaptor strategy [21]. This library had 5×10^5 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 2×10^7 Kn^R 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 *GAL* 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 recombination *in vivo*. 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 manipulaion 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 (>10^8 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 recombination-based cloning technologies overcomes many of the defects associated with the currently available conventional cloning methods. Together, these methods constitute a comprehesive strategy for the rapid and systematic generation and manipulation of recombinant DNA molecules without the use of restriction enzymes. Furthermore, these recombination-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 [*Δlac-169*, *rpoS(am)*, *robA1*, *creC510*, *hsdR514*, *uidA(ΔMluI)::pir-116*, *endA*, *recA1*] and BW23473[*Δlac-169*, *robA1*, *creC510*, *hsdR514*, *uidA(ΔMluI)::pir*$^{+}$, *endA*, *recA1*] [24] were a gift of B. Wanner and were used as hosts for propagation of all univector based plasmids. CX1 (*slyD*) was used for propagation of *E* expressing plasmids [14]. JM107 or DH5α was used as recipient host for UPS reactions. BUN10 [*hisG4 thr-1 leuB6 t lacY1 kdgK51 Δ(gpt-proA)62 rpsL31 tsx33 supE44 recB21 recC22 sbcA23 hsdR::cat-pir-116*(Cm^{R})] was used for homologous recombination experiments. BUN13, which has *cre* under the control of the *lac* promoter, is JM107 lysogenized with λ_{LC} (*aadA lac-cre*). BUN15 is XL1 blue containing pML66 (*tac-R*, Sp^{R}) and was used for the *in vivo* R recombination assays. *E. coli* BL21 was used for bacterial expression studies. *S. cerevisiae* Y80 [25] was used for yeast expression studies and Hi5 cells were used for baculovirus expression. Y543 (as Y80 but *cdc4-1*) was used for *cdc4* suppression [22].

Construction of BUN13

The pSE356 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 *Bam*HI–*Hin*dIII (blunt-ended) fragment containing *aadA* (Sp^{R}) from pDPT270 [26] was subcloned into *Bam*HI–*Sph* I (blunt-ended) pSE356 to create pQL102, replacing *neo* with *aadA*. Then a PCR-amplified *cre* gene (5′-GCGGCCGCTGAGTGTTAAATGTCCAATT-3′ and 5′-CCCGGGCTAATCGCCATCTTCCAGC-3′) was cloned into pCR^{TM}II (Invitrogen) and subcloned as a *Not*I–*Eco*RI fragment into pQL102, placing *cre* under *lac* control adjacent to *aadA* and flanked by λ DNA. The λ_{KC} phage [21] was amplified on JM107 containing pQL114 and the resulting phage lysate containing the desired recombinant λ_{LC} phage was used to infect JM107. Sp^{R} Kn^{S} 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 *Nco*I site at the first ATG using primers 5′-CCATGGCCAATTTACTGACCGTACAC-3′ and 5′-CCCGGGCTAATCGCCATCTTCCAGC-3′. The PCR product was cloned into pCR^{TM}II and subcloned into pGEX-2Tkcs (W.J. Harper, unpublished) as an *Nco*I–*Eco*RI fragment to create pQL123. The pHOST plasmid pQL103 was made by deleting one of two *loxP* sites from pSE1086 (S.J.E., unpublished) between *Not*I and *Sal*I. The 590 bp *Nco*I–*Bam*HI *SKP1* ORF was subcloned from pCB149 [11] into *Nco*I–*Bam*HI-cleaved pUNI10 to create pQL130 (pUNI10-SKP1) The pUNI-RAD53 (pQL135) clone was constructed by subcloning the HA_3–*RAD53* gene as an *Nco*I–*Not*I (blunt-ended) fragment from pQL49 into *Nco*I–*Bam*HI (blunt-ended) pUNI10. The MYC_3–*RNR4* gene was subcloned from pMH176 [27] into pUNI10 to create pQL248, or into pBAD104 (B. Desaney, unpublished) to create the control *GAL:*MYC_3–*RNR4* plasmid pQL222. The pQL138 and pQL193 plasmids were derived from pBAD104 by insertion of oligonucleotide linkers containing *loxP* (5′-TCGAGACGTCATAACTTCGTATAGCATACATTATACGAAGTTATGC-3′ and 5′-GCCGCATAACTTCGTATAATGTATGCTATACGATGTTATGACGTC-3′ or *loxH* (5′-CATGGCTATAACTTCGTATAGCATACATTATACGAAGTTATG-3′ and 5′-GATCCATAACTTCGTATAATGTATGCTATACGAAGTTATAGC-3′), respectively. Two *GAL:*MYC_3–*RNR4* constructs were made by UPS using pQL248 with pQL138 or pQL193. The construction details of pHB2-GST, pHY314-MYC_3, pHY326, pHI100-GST, pHI100-MYC_3, pHI100-HA_3 and pHM200-HA_3 are available upon request. The pQL269 plasmid (*lac–cre,* Sp^{R} and ori^{Ts}) was constructed by ligating the *Eco*RI–*Pvu*II fragment from pQL114, containing *aadA* (conferring spectinomycin resistance) and the *lac–cre* chimeric gene, to a *Bgl*I(blunt)–*Eco*RI fragment containing the temperature-senstive replication origin from pINT-ts [28]. The pML66 was constructed by ligating the *Eco*RI–*Sal*I (blunt-ended) fragment containing the *tac* promoter driving the R recombinase from pNN115 [13] to *Eco*RI–*Pst*I (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 *Nco*I and *Sal*I sites of pAS2 [15] followed by a three-way ligation using a *Xho*I–*Spe*I fragment containing *tac-E*, a *Spe*I–*Pst*I synthetic *RS* oligonucleotide linker, and *Sal*I–*Pst*I linearized pAS2-lox. The pML74 plasmid was made by first replacing the *neo* gene on pUNI20 with the *bla* gene from pUC19 and subsequently inserting the MYC_3 tag that was PCR amplified from pJBN48 (J. Bechant, unpublished; details upon request). The PCR primers used to amplify MYC_3 from pML74 for the 3′ tagging of *SKP1* were primer A (MZL179) 5′-CCAGAGGAGGAGGCTGCCATTAGGCGTGAAAATGAATGGGCTGAAGACCGTTCTGAGCAAAAGCTCATTTC-3′ and primer B (MZL161) 5′-GGATATAGTTCCTCCTTTCAGC-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 OD_{600} 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 × buffer S (50 mM Tris HCl (pH 7.5); 10 mM $MgCl_2$, 30 mM NaCl, 0.1 mg/ml BSA). The reaction mixtures were assembled on ice and incubated at 37°C for 20 min followed by incubation at 65°C for 5 min to inactivate GST–Cre. Typically half of the reaction mixture was transformed 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 spectinomycin and IPTG and grown to OD_{600} of 0.5 at 30°C, and electrocompetent cells were prepared as recommended (BioRad). In each transformation, 40µl competent cells were used, and after the electroporation 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 µg of each plasmid was used for electroporation followed by incubation with LB (0.5% glucose) media at 37°C for 1 h. The transformants were plated at different dilutions onto LB (0.5% glucose) plates 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.

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 incubated at 30°C for 2 h. β-galactosidase activities were measured as previously described [25].

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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Supplementary material

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

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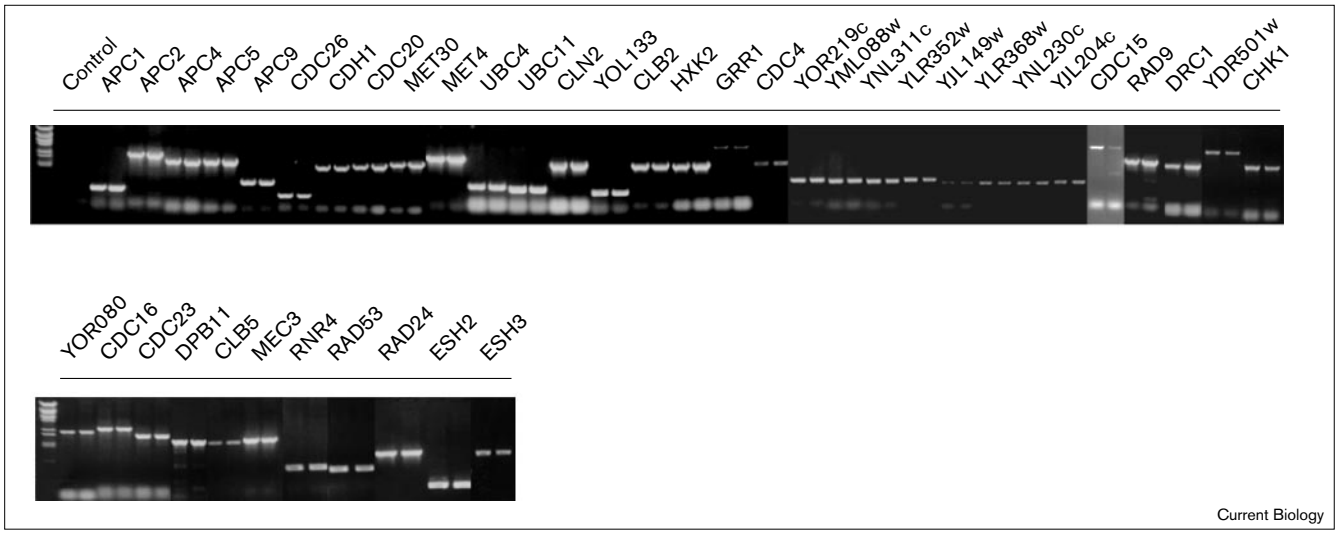
Figure S1



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.