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IpO: Plasmids and methods for simplified, PCR-based DNA transplant in yeast

Joe Horecka*, Angela M. Chu, and Ronald W. Davis

Department of Biochemistry, 318 Campus Drive, Clark Center W300, Stanford University, Stanford, CA 94305, USA

Abstract

Many yeast experiments require strains modified by recombinant DNA methods. Some experiments require precise insertion of a DNA segment into the genome without a selectable marker remaining. For these applications, we developed a new PCR-based method for marker-free DNA transplant. The current PCR-based method requires the labor-intensive construction of a PCR template plasmid with repeats of the DNA segment flanking *URA3*. The design of a new vector, IpO, reduces the work to cloning a single copy of the DNA segment between overlapping *URA3* fragments present in the vector. Two PCRs are performed that capture the DNA segment and one or the other *URA3* fragment. When the PCR products are co-transformed into yeast, recombination between the overlapping *URA3* fragments restores *URA3* and transposes the cloned DNA segment inside out, creating a repeat-*URA3*-repeat cassette. Sequences designed into the PCR primers target integration of the cassette into the genome. Subsequent selection with 5-fluoro-orotic acid yields strains that have popped out *URA3* via recombination between the DNA repeats, with the result being the precise insertion of the DNA segment minus the selectable marker. An additional advantage of the IpO method is that it eliminates PCR artifacts that can plague the current method's repeat-containing templates.

Keywords

yeast; PCR; marker-free; DNA insertion

INTRODUCTION

Many yeast experiments require strains modified by insertion of a DNA segment at a specific site in the genome. In some experiments, such as deleting a gene by one-step gene replacement, the DNA segment serves only as a selectable marker for transformation. In other experiments, the DNA segment plays a wider role in the experimental analysis. Examples of this class of DNA segments include transcriptional regulatory elements, transcriptional reporters, and recombinant tags for protein detection. In this report, we focus on the latter class of DNA segments, and in particular the methods used to insert them at specific sites in the genome. The DNA segment can be derived from yeast, another organism, or be purely synthetic. Because the methods entail the transfer of the DNA

*Correspondence to: jhorecka@stanford.edu. Tel and fax (650) 725-3635.

segment from its source to the genome of the host yeast, we refer to process as DNA transplant.

Two common approaches for transplanting DNA segments are one-step methods that retain the selectable marker used for transformation and two-step methods that can result in marker-free DNA insertion. The choice of which to use is dictated by the particular experiment. Although marker-free DNA transplant requires significantly more work, some experiments demand it. Examples include inserting a transcriptional regulatory element to place a gene under heterologous control while avoiding effects that might be associated with the selectable marker, inserting DNA encoding a fluorescent tag within an open reading frame (ORF), and constructing a strain with several modifications that would make the accumulation of selectable markers impractical.

The original method for marker-free DNA insertion requires two steps and is referred to as “pop-in/pop-out” gene replacement [Scherer and Davis, 1979]. A length of genomic target DNA is cloned into an integrating plasmid containing the selectable/counters selectable *URA3* marker. Next, the DNA segment that will be transplanted into the genome is inserted into the cloned DNA by site-specific mutagenesis. The first step is transformation of yeast with the new plasmid that has been cut at a restriction site within the cloned DNA to target “pop-in” integration. Transformation with a circular plasmid produces on the chromosome a direct repeat of the cloned genomic DNA separated by the *URA3* plasmid, with the inserted DNA segment on one side and wildtype on the other. The second step is initiated by culturing cells in the absence of uracil selection. At a low frequency, cells arise in the population that have undergone recombination between the direct repeats flanking the *URA3* plasmid. These rare “pop-out” recombinants are selected on 5-fluoro-orotic acid (FOA) medium, which is toxic to *URA3* cells [Boeke *et al.* 1984]. The resulting strains retain either the inserted DNA segment or wildtype DNA, depending on where the pop-out crossover occurred. The original method, although elegant, is cumbersome because it is a multi-week process that requires construction of two new plasmids for each desired chromosomal change.

There is a PCR-based method available that simplifies marker-free DNA transplant in yeast [Schneider *et al.*, 1995; Khmelinskii *et al.*, 2011]. It is also a two-step method and uses the basic principles of the original: transformation with linear DNA that has yeast sequences on each end to target pop-in integration, and FOA selection for pop-out recombination between direct DNA repeats flanking *URA3*. A clever twist of the PCR-based method is that the DNA segment itself becomes the repeated DNA that flanks *URA3*. Because of this reconfiguration, the only plasmid that needs to be made is a PCR template that contains, in this order, the DNA segment, *URA3*, and a second copy of the DNA segment (repeat-*URA3*-repeat, see for example Figure 1A). To create a repeat-*URA3*-repeat cassette for yeast transformation, a PCR is performed using the plasmid template and primers that contain both yeast sequences to target integration and plasmid sequences to prime PCR.

The PCR-based method simplifies DNA transplant by eliminating the need to construct two plasmids for each genomic target. Another advantage is that having the DNA segment as the direct repeat means that all pop-out recombinants retain the DNA segment. Nonetheless, the method has room for improvement. Construction of the repeat-*URA3*-repeat PCR template

plasmid is labor-intensive, requiring either a four-part ligation or construction of three sequential plasmids made by two-part ligation. The work is multiplied when one wishes to make template plasmids for a variety of DNA segments. Moreover, PCR amplification of repeat-containing templates is problematic, producing artifacts or failing altogether.

In a study to interrogate long non-coding RNA (ncRNA) function in yeast, we wished to place a set of ncRNAs under heterologous transcriptional control by inserting a variety of promoters and terminators near ncRNA transcription start sites (TSS). Because ncRNAs are often close to or overlap other annotated genomic features (e.g. see [Lardenois *et al.*, 2011]), it is essential to use marker-free DNA transplant to insert the transcriptional control elements. Our first approach was to use the existing PCR-based method. However, while constructing and using PCR template plasmids, we encountered the problems noted above: labor-intensive plasmid construction and problematic PCRs using repeat-*URA3*-repeat templates. In this report, we describe how we solved both problems by creating a new PCR template vector and by reconfiguring the PCRs used to make DNA for yeast transformation.

MATERIALS AND METHODS

Standard techniques were used for DNA manipulations [Ausubel *et al.*, 1995]. DNA oligonucleotides (Table 1) were synthesized by Eurofins MWG Operon (Huntsville, AL). We used the following PCR polymerases: Phusion Hot Start II High Fidelity (Thermo Scientific) to amplify DNA that would be cloned or directly sequenced, Takara ExTaq to amplify DNA for yeast transformation, and Phire Hot Start II (Thermo Scientific) for genomic confirmation PCR. Cloned DNA segments were sequenced in their entirety, except where noted for pJH104. Yeast media, culture conditions, and molecular techniques were as described by [Amberg *et al.*, 2005]. Assay of α -factor secretion was as described by [Sprague, 1991].

PCR template plasmid pJH104 (Addgene 48262) carries two copies of the *TEF1* promoter flanking *URA3*. U2 and D2 sequences provide priming sites for PCR [Chu and Davis, 2008]. pJH104 was constructed as follows. The *XhoI*-U2-*TEF1p*-*XbaI* segment was PCR amplified from NKY210 [Raymond and Kleckner, 1993] genomic DNA with primers TEF1.34.1 and TEF1.34.2; *TEF1p* sequences are from -460 to -128 relative to the *TEF1* start codon. *XbaI*-*URA3*-*BamHI* was amplified from pRS316 [Sikorski and Hieter, 1989] with primers URA3.34.1 and URA3.34.2. The *BamHI*-*TEF1p*-D2-*EagI* segment was amplified from NKY210 with primers TEF1.34.3 and TEF1.34.4; *TEF1p* sequences are from -343 to -1. The three DNA segments were digested with restriction enzymes and combined in a four-part ligation with *XhoI* and *EagI* digested pBluescript II SK (-) (Agilent Technologies). The cloned *TEF1p* segments and most of *URA3* were confirmed by DNA sequencing. The *TEF1p* segments share 215 bp overlap. When the *TEF1p*-*URA3*-*TEF1p* cassette is integrated in yeast, recombination between the *TEF1p* repeats “pops-out” the intervening *URA3* marker and restores the full-length, 460 bp *TEF1* promoter.

IpO (Addgene 48233) is the base vector for building DNA transplant plasmids. It carries overlapping *URA3* fragments flanking a multiple cloning site (Figure 2). IpO was constructed in two steps. First, the *URA3* 5' fragment was PCR amplified from JHY222

genomic DNA (S288c background, [Lardenois *et al.*, 2011]) with primers URA3.39.3 and URA3.39.4, which add *Xho*I and *Kpn*I sites; *URA3* sequences are from –242 to +495. The resulting product was digested with *Xho*I and *Kpn*I and inserted into the same sites of pBluescript II SK (–) to create pJH121. Next, the *URA3* 3′ fragment was likewise amplified with primers URA3.39.1 and URA3.39.2, which add *Sac*I and *Eag*I sites; *URA3* sequences are from +216 to 80 bp downstream of the stop codon. The resulting product was digested with *Sac*I and *Eag*I and inserted into the same sites of pJH121, creating IpO. The *URA3* fragments share 280 bp overlap.

We created two promoter transplant plasmids based on IpO. Each has UP and DN priming sites flanking the DNA insert. Thus, either promoter can be transplanted using a single pair of gene-specific primers with UP and DN sequences on their 3′ ends. pJH124 (Addgene 48259) carries the 284 bp *Ashbya gossypii TEF1* promoter. It was created by PCR amplification of the *Ag TEF1* promoter from pFA6a-kanMX4 [Wach, *et al.*, 1994] with primers aTEF1.39.1 and aTEF1.39.2, which add *Xba*I plus UP before *Ag TEF1p*, and DN plus *Xho*I after. The resulting product was digested with *Xba*I and *Xho*I and cloned into the same sites of IpO. The *TEF1* promoter sequences in pJH124 differ from those found in common vectors. pJH124 contains exactly the intergenic region between the tandem-transcribed *A. gossypii MRL1* and downstream *TEF1* genes. The *TEF1* promoter in pFA6-kanMX4 adds 95 bp of the *MRL1* ORF and leaves out 2 bp before the *TEF1* start codon. Our *TEF1* promoter may be advantageous because it is shorter (284 vs. 378 bp) and contains all sequences prior to *TEF1*. Based on the tandem orientation of *MRL1* and *TEF1*, the *TEF1* promoter is likely unidirectional. In pJH124, the *TEF1* promoter is oriented towards the DN sequence and the *URA3* 5′ fragment. Plasmid pJH131 (Addgene 48260) carries the 668 bp *S. cerevisiae GAL10/GAL1* bidirectional promoter, corresponding to the entire sequence between the two divergent genes. It was created by PCR amplification of *GAL10/GAL1* promoter DNA from JHY222 with primers GAL50.1 and GAL50.2, which add *Nhe*I and *Sac*II sites. The resulting product was digested with *Nhe*I and *Sac*II and cloned into the same sites of pJH124, which are present in the UP and DN sequences, respectively. The *GAL1* promoter is oriented towards the DN sequence and the *URA3* 5′ fragment.

We used the IpO method to create several *MAT α* strains with either the *TEF1* or *GAL1* promoter transplanted upstream of the *MAT α* -specific *BAR1* gene. In one set of strains the promoters were used to replace the *BAR1* upstream control region from –278 to –1 [Kronstad *et al.*, 1987]. Transplant of the *Ag TEF1p* promoter is illustrated in Figure 3. Two split-*URA3* PCRs (Figure 4A) were performed for each pJH124 and pJH131 using the following primer pairs: BAR1.61.2 and URA3.rev for the promoter-*URA3* 5′ fragment, and URA3.for and BAR1.61.3 for the *URA3* 3′-promoter segment. In another set of strains the promoters were inserted directly upstream of the *BAR1* start codon without replacing any DNA. The split-*URA3* PCRs were the same as for the promoter replacement PCRs above, except that the promoter-*URA3* 5′ fragment was amplified with BAR1.61.1 and URA3.rev primers. Split-*URA3* PCR pairs were mixed 1:1 (22.5 μ L each, ~7 μ g total DNA) and used without concentration or purification to transform JHY337 (*MAT α ura3⁰ leu2⁰ lys2⁰*; [Horecka and Davis, 2014]) using the method of [Gietz and Schiestl, 2007]. One fifth of the transformations were spread on SC-Ura plates, which were replica plated to fresh plates

after two days to eliminate the background lawn that is often present with PCR transformations. Over 100 colonies were obtained for each transformation. Correct transformants were identified by genomic PCR using primers specific to one side of the integrated *URA3* cassette. For the heterologous *Ag TEF1* promoter, 16 of 16 scored as correct. For the *Sc GAL1* promoter, 10 of 80 scored as correct. Several independent transformants were streak purified on YPD plates and then a single colony of each was used to inoculate 3 mL YPEG broth (2.5% ethanol, 2% glycerol; used to prevent growth of petites). Cultures were grown to saturation ($\sim 2 \times 10^8$ cells/mL) for 2 days at 30°C. Twenty microlitres culture fluid ($\sim 4 \times 10^6$ cells) was spread onto a plate of minimal complete medium containing 0.8 mg/mL FOA (US Biological). An average of 114 colonies arose on each plate (range 5 to ~ 200 colonies, six plates scored).

RESULTS

In a study to be published elsewhere, we wished to alter the expression of many long ncRNAs at their genomic loci. A class of ncRNAs of particular interest are those that are close to or overlap with genes of known function (e.g. see [Lardenois *et al.*, 2011]), which makes them a challenge for heterologous regulation. One method we considered was PCR-based, one-step promoter insertion to place a *kanMX6-PGAL1* promoter cassette at the ncRNA TSS [Longtine *et al.*, 1998]. Although this method is amenable to the study of many ncRNAs, we were concerned about off-target effects on nearby genes that might be caused by the *kanMX6* marker. Ideally, a minimal regulatory DNA segment could be inserted at an ncRNA TSS without a marker or other DNA remaining. One method that can accomplish this is classical two-step gene replacement [Scherer and Davis, 1979]. This method requires that new plasmids be built for each target. Because we wanted to study many ncRNAs, the plasmid-based method was not a practical option. Instead, we chose as a first approach to adapt a PCR-based, two-step method that has been used to introduce marker-free epitope and fluorescent tags into genomic coding sequences [Schneider *et al.*, 1995; Khmelinskii *et al.*, 2011].

The present PCR-based DNA insertion method requires construction of a PCR template plasmid containing two repeats of the DNA to be inserted flanking *URA3*. The repeat-*URA3*-repeat construct also has priming sites that allow PCR amplification of the entire cassette using primers that include genomic targeting sequences [Schneider *et al.*, 1995; Khmelinskii *et al.*, 2011]. We used this base architecture to construct pJH104, which has repeats of the *S. cerevisiae TEF1* promoter instead of sequences encoding a recombinant protein tag (Figure 1A). As a proof of principle for promoter transplant using pJH104, we chose to target *BAR1*, which encodes the secreted α -factor protease for which there is a convenient plate assay. When attempting to PCR amplify the entire cassette for transformation using *BAR1.U2* and *BAR1.D2* primers, we analyzed the reaction on a gel and found not only the expected full-length product, but also a shorter PCR product (Figure 1C). The shorter product appeared with all of the PCR polymerases we tried (ExTaq, Phusion Hot Start II, Phire Hot Start II, Invitrogen Platinum Taq, Agilent Herculase II), and even with the optimized PCR conditions recommended for similar repeat-containing templates [Khmelinskii *et al.*, 2011]; some of our repeat-*URA3*-repeat PCRs failed altogether (data not shown). To determine the structure of the smaller product, we cloned

and sequenced it. Two independent clones had the structure of BAR1-U2-repeat-D2-BAR1 (illustrated in Figure 1B, data not shown), which suggests melting and reannealing of nascent PCR products during the extension step. Our finding of PCR artifacts when amplifying from a repeat-*URA3*-repeat template is not unique [de Hoogt *et al.*, 2000; Khmelinskii *et al.*, 2011; Schneider *et al.*, 1995].

We wanted to eliminate the PCR artifact because it reduces desired product yield and may reduce transformation efficiency. We took advantage of the fact that selectable markers split into overlapping fragments can be co-transformed into yeast and recombine with each other to restore a full-length, functional marker [Fairhead *et al.*, 1996]. With this in mind, we designed two *URA3* primers that could be used individually with primers BAR1.U2 and BAR1.D2 and template pJH104 to create PCR products that share 215 bp *URA3* overlap (Figure 1A). The product of one PCR was predicted to be *BAR1* sequences, *TEF1*p, and the *URA3* 5' fragment (PCR 2, Figure 1B). The other was predicted to be the *URA3* 3' fragment, *TEF1*p, and *BAR1* sequences (PCR 3, Figure 1B). Gel analysis showed robust amplification of DNA molecules of the expected sizes and, notably, none of the artifact product seen with the single PCR (Figure 1C). Thus, separating the repeats into two PCRs solved the artifact problem. The pair of split-*URA3* products were mixed and used to transform yeast. After identifying correct integrants, cells were grown in the absence of uracil selection to allow for recombination between the *TEF1*p repeats and pop out of *URA3*. Following selection on FOA plates, strains were isolated and confirmed by genomic PCR, DNA sequencing, and plate assay to have the *TEF1* promoter inserted upstream of *BAR1* (data not shown).

We learned two lessons from constructing and using pJH104 for *TEF1*p promoter transplant. First, creating a repeat-*URA3*-repeat PCR template plasmid is not trivial. We built pJH104 by combining three DNA segments with the vector in a four-part ligation. An alternative approach requires three sequential, two-part plasmid constructions. Laborious plasmid construction can be a bottleneck if one wants to build many PCR template plasmids. Second, PCR amplification of repeat-*URA3*-repeat templates is problematic, producing an artifact product that lowers desired product yield or failing altogether. By reconfiguring PCR template plasmid design and the protocol for cassette amplification, the method can be greatly simplified and made more robust.

The ability of split-*URA3* PCRs to remedy the problematic amplification of repeat-*URA3*-repeat cassettes provided the foundation for reconfiguring PCR template plasmid design. If two separate PCRs are required, then there is no need to build a complicated repeat-*URA3*-repeat plasmid. The template requirements then become: 1) the DNA segment cloned upstream of a *URA3* 5' fragment and 2) the DNA segment cloned downstream of a *URA3* 3' fragment. Instead of building two independent vectors [Fairhead *et al.*, 1996; Reid *et al.*, 2002], we incorporated both requirements into a single vector. Plasmid IpO contains both *URA3* 5' and *URA3* 3' fragments flanking a multiple cloning site (Figure 2). The DNA segment that one wishes to transplant into the genome is cloned between the overlapping *URA3* fragments in a simple, two-part ligation. The resulting plasmid is then used for split-*URA3* PCRs, with one reaction amplifying the cloned DNA coupled to the *URA3* 5' fragment, and the other amplifying the *URA3* 3' fragment coupled to the cloned DNA

(Figure 3). When the products are co-transformed into yeast and recombine, the cloned DNA segment that was located inside the *URA3* fragments in the plasmid is now repeated and transposed outside the restored *URA3* marker (inside-out, “IpO”). Adding genomic sequences to the 5′ ends of two of the split-*URA3* PCR primers allows targeted integration of the repeat-*URA3*-repeat cassette.

As a proof of principle for the IpO vector and method, we again chose to target the *BAR1* gene. We built two promoter transplant plasmids based on IpO. Plasmids pJH124 and pJH131 carry the *A. gossypii TEF1* and *S. cerevisiae GAL10/GAL1* promoters, respectively. We added UP and DN primer binding sites flanking the promoters to allow transplant of either promoter with the same set of *BAR1* primers. One approach was to replace the *BAR1* upstream control sequences (−278 to −1) with the promoters. The method for transplanting *TEF1p* in place of the *BAR1* upstream sequences is shown in Figure 3. Transplanting the *GAL10/GAL1* promoter is the same, except the template is pJH131 instead of pJH124. A second approach was to insert the promoters upstream of the *BAR1* start codon without replacing any sequences. Split-*URA3* cassettes were amplified by PCR (Figure 4A) and used to transform *MAT α* cells, which do not express *BAR1*. Following transformation, screening for correct integrants, and selecting for *URA3* pop-out strains on FOA plates, we identified correct promoter transplant strains of each type, which were verified by genomic PCR (Figure 4B) and DNA sequencing (data not shown). Halo assays to detect barrier activity confirmed the correct pattern of heterologous *BAR1* regulation (Figure 4C). In this assay, secreted α -factor inhibits growth of the *MAT α bar1* lawn, creating a halo (e.g. wt in Figure 4C). Simultaneous expression of barrier protease in the same *MAT α* cell inactivates α -factor, and therefore the lawn is not affected. The constitutive *TEF1* promoter directed *BAR1* expression on both YPDextrose and YPGalactose plates, as expected (Figure 4C). Likewise, the regulatable *GAL1* promoter showed the correct pattern of *BAR1* expression, with barrier being expressed on YPGalactose plates, but not on YPDextrose plates (Figure 4C). We found no observable difference between replacing the *BAR1* upstream sequences and simply inserting the promoters before the start codon. This result was not necessarily expected, because the α 2-Mcm1 operator located between −264 and −235 upstream of *BAR1* has been shown to have a dominant, repressive effect when placed between heterologous *CYC1* UAS and TATA promoter sequences [Zhong and Vershon, 1997]. It is interesting that even the shorter 284 bp *Ag TEF1* promoter inserted before the *BAR1* start codon was not measurably repressed by the presence of the α 2-Mcm1 operator.

DISCUSSION

Here we have described the IpO vector and methods for simplified, PCR-based DNA transplant in yeast. Two features that set it apart from the existing PCR-based method are the IpO base vector and the use of split-*URA3* PCR to amplify DNA for yeast transformation. The IpO vector streamlines construction of PCR template plasmids by requiring only a two-part ligation. This is a significant improvement over the complicated assembly of repeat-*URA3*-repeat template plasmids in the present method. The design of IpO places the cloned DNA segment both upstream of a 5′ *URA3* fragment and downstream of a 3′ *URA3* fragment. Two separate, split-*URA3* PCRs from the same template produces

DNA molecules that recombine upon yeast transformation to create direct repeats of the cloned DNA segment flanking an intact *URA3* selectable marker. It is both the unique design of IpO with its split *URA3* fragments and the efficient homologous recombination system in yeast that make the method possible.

Another improvement of the IpO method is the use of split-*URA3* PCR to prepare DNA for transformation. Single PCRs containing repeat-*URA3*-repeat templates are problematic. Our failed attempts to remedy the problem by trying different PCR polymerases and reaction conditions motivated us to reconfigure the strategy for PCR amplification of DNA from template plasmids. We found that split-*URA3* PCR using template pJH104 is robust and free of detectable artifact products. This result, in turn, inspired us to reconfigure template plasmid design, resulting in the creation of the IpO vector. Because split-*URA3* PCR with IpO-based plasmids uses two universal *URA3* primers, transplanting a DNA segment to a new locus requires only two new genome-targeting primers. Thus, the method is also economical.

In our proof of principle experiment we used the IpO method to transplant the *Ag TEF1* and *Sc GAL10/GAL1* promoters upstream of *BAR1*. The method, of course, can be used to transplant virtually any DNA segment. Examples include transcription terminators, reporters such as *lacZ* or *HIS3*, epitope tags, fluorescent tags, and purification tags. For economy, we incorporated universal UP and DN primer sites flanking the *Ag TEF1* and *GAL10/GAL1* promoters so that we could use the same *BAR1* primers to amplify both. This approach leaves the UP and DN 18 bp sequences flanking the inserted DNA in the final strain, which is probably acceptable for promoter and terminator transplant. However, there are cases in which seamless DNA transplant is required, such as the insertion of an epitope tag within an ORF. In such cases, genomic-targeting primers can be designed with 3' ends that hybridize to both sides of the DNA segment cloned into IpO. This is not an option with methods based on exact repeat-*URA3*-repeat templates, because there would be two forward and two reverse primer-binding sites on the plasmid that would lead to additional problems with PCR.

In summary, we have developed a novel method for PCR-based DNA transplant in yeast. The IpO vector is unique in that it is designed with split *URA3* fragments flanking the multiple cloning site and that it takes advantage of the efficient homologous recombination system in yeast to reconstruct an intact *URA3* marker. The IpO method is an improvement over current PCR-based methods because it simplifies construction of PCR template plasmids and allows for problem-free, robust amplification of DNA for yeast transformation. The IpO vector (48233) and the two promoter transplant plasmids pJH124 (*Ag TEF1p*, 48259) and pJH131 (*Sc GAL10/GAL1p*, 48260) are available from Addgene.

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Figure 1.

PCR amplification from a repeat-*URA3*-repeat template. (A) pJH104, a *TEF1p-URA3-TEF1p* plasmid. U2 and D2 priming sequences flank the cassette. Primers URA3.34.7 and URA3.34.8 are used for split-*URA3* PCRs. (B) PCRs with different primer combinations. The *BAR1* sequences (grey bars) have 60 bp genomic sequence followed by U2 or D2. PCR products are illustrated below the three PCRs. The 556 bp artifact product in PCR 1 corresponds to *BAR1-U2-TEF1p-D2-BAR1*, as determined by cloning and sequencing of similar products. (C) Gel analysis of the three PCRs shown in (B).

Figure 2.

IpO, a vector for building DNA transplant plasmids. The *URA3* fragments share 280 bp overlap (ORF coordinates +216 to +495). The multiple cloning site has nine unique restriction sites.

Figure 3.

Overview of the IpO method for DNA transplant. Illustrated is the replacement of *BAR1* regulatory sequences with the *Ag TEF1* promoter. (A) A portion of IpO-based plasmid pJH124, which contains the 284 bp *Ag TEF1* promoter flanked by UP and DN priming sites. The two split-*URA3* PCRs use primer pairs BAR1.61.2 + *URA3.rev* and *URA3.for* + BAR1.61.3. (B) Recombination and integration (dotted lines, “pop-in”) of the split-*URA3* PCRs from (A) at the genomic *BAR1* locus (coordinates relative to start codon). The grey bars attached to the *Ag TEF1p* (T) sequences are the 60 bp *BAR1* sequences designed into the primers. (C) Structure of the genomic *BAR1* locus, with the *Ag TEF1p-URA3-Ag TEF1p* cassette replacing precisely *BAR1* sequences from -278 to -1. The dotted line indicates the directly repeated *Ag TEF1p* segments that can undergo homologous recombination (“pop-out”) to yield the product shown in (D), which is the *Ag TEF1* promoter precisely transplanted upstream of the *BAR1* ORF.

Figure 4.

Use of the IpO method for heterologous regulation of *BARI* in *MATα* cells. Numbers correspond to: 1, *Ag TEF1p* inserted at *BARI* start (@ATG); 2, *Ag TEF1p* replacing *BARI* UAS (@UAS); 3, *GALI*p inserted at *BARI* start; 4, *GALI*p replacing *BARI* UAS. (A) Split-*URA3* PCR amplification of DNA for transformation. Primer pairs are *BAR1.61.1* (or *BAR1.61.2*) + *URA3.rev* (left) and *URA3.for* + *BAR1.61.3* (right). Marker (M) sizes for (A) and (B) are given in kilobasepairs (kb); the bright band is 3.0 kb. (B) Genomic PCR of wildtype (wt) *BARI* and promoter transplant strains following FOA selection. Expected sizes (bp): wt (649); 1, *Ag TEF1p*@ATG (969); 2, *Ag TEF1p*@UAS (691); 3, *GALI*p@ATG (1353); 4, *GALI*p@UAS (1075). (C) Heterologous expression in *MATα* cells of barrier, the α -factor protease. Halo assays were performed by suspending *MATα bar1* cells in water and spreading on YPGalactose (Gal) and YPDextrose (Dex) plates. *MATα* cells were taken from Gal or Dex stock plates and patched onto the lawns. Plates were incubated at 30C and photographed after one (Dex) or two (Gal) days. Secreted α -factor inhibits growth of the *MATα bar1* lawn, creating a halo (e.g. wt). Simultaneous expression of barrier protease in the same cell inactivates α -factor.

Table 1

PCR Primers

Name	Purpose	Sequence (5' to 3')
TEF1.34.1	Forward <i>XhoI</i> -U2- <i>TEF1</i> p- <i>XbaI</i> , pJH104	TTCCTCGAGCGTACGCTGCAGGTCGACGA ATCCTTACATCACACCCAATC
TEF1.34.2	Reverse <i>XhoI</i> -U2- <i>TEF1</i> p- <i>XbaI</i> , pJH104	GGTTCTAGAATCAATGGGAGGTCATCGAA AG
URA3.34.1	Forward <i>XbaI</i> - <i>URA3</i> - <i>BamHI</i> , pJH104	GGTTCTAGAAGAGTGCACCACGCTTTTCAA T
URA3.34.2	Reverse <i>XbaI</i> - <i>URA3</i> - <i>BamHI</i> , pJH104	CGCGGATCCTTTACACCCGAGGGTAATA AC
TEF1.34.3	Forward <i>BamHI</i> - <i>TEF1</i> p-D2- <i>EagI</i> , pJH104	GGTTCTAGACGCGGATCCCACCTCAAAAC ACCCAAGCACA
TEF1.34.4	Reverse <i>BamHI</i> - <i>TEF1</i> p-D2- <i>EagI</i> , pJH104	CTTCGGCCGTCATCGATGAATTCGAGCTC GTTTGTAAATTAATACTTAGATTAGATTGC TATGCTTTC
URA3.34.7	Reverse split- <i>URA3</i> PCR, pJH104	AGTATATTCTCCAGTAGATAGGGAGCC
URA3.34.8	Forward split- <i>URA3</i> PCR, pJH104	TGCTGCTACTCATCCTAGTCCTG
BAR1.U2	60 nt homology upstream of <i>BAR1</i> ATG + U2	AGTGGTTCGTATCGCCTAAAATCATACCA AAATAAAAAGAGTGTCTAGAAGGGTCAT ATACGTACGCTGCAGGTCGAC
BAR1.D2	Reverse complement of 60 nt homology from <i>BAR1</i> ATG to +60 of ORF + D2	GTTAATAATCGCGAAACTCGCCAAAATAA GTTTCAAACAAAGATGATTAATTGCAGAC ATATCGATGAATTCGAGCTCG
URA3.39.3	Forward <i>XhoI</i> - <i>URA3</i> 5'- <i>KpnI</i> , pJH121	TTCTCTGAGTGTGGTTTCAGGGTCCATAAA G
URA3.39.4	Reverse <i>XhoI</i> - <i>URA3</i> 5'- <i>KpnI</i> , pJH121	GTGGGTACAGTATATTCTCCAGTAGCTA GGGAGCC
URA3.39.1	Forward <i>SacI</i> - <i>URA3</i> 3'- <i>EagI</i> , IpO	AGGGAGCTCACAGTTAAGCCGCTAAAGGC ATTA
URA3.39.2	Reverse <i>SacI</i> - <i>URA3</i> 3'- <i>EagI</i> , IpO	CTTCGGCCGGTAATAACTGATAT AATTAAATTGAAGC
aTEF1.39.1	Forward <i>XbaI</i> -UP-AgTEF1p-DN- <i>XhoI</i> , pJH124	GGTTCTAGACCGGTGCATGCGCTAGCTGTG ACTGTCGCCGTACAT
aTEF1.39.2	Reverse <i>XbaI</i> -UP-AgTEF1p-DN- <i>XhoI</i> , pJH124	TTCTCGAGGACGTCTGTACACCGCGGTTT TGTTTATGTTCCGATGTGATGTG
GAL1.50.1	Forward <i>NheI</i> - <i>GALp</i> - <i>SacII</i> , pJH131	AAAAGCTAGCTTATATTGAATTTTCAAAA ATTCTTACTTTTTTTT
GAL1.50.2	Reverse <i>NheI</i> - <i>GALp</i> - <i>SacII</i> , pJH131	AAAACCGCGGTATAGTTTTTCTCCTTGAC GTAAAGTATAGAGG
BAR1.61.1	60 nt homology upstream of <i>BAR1</i> ATG + UP	AGTGGTTCGTATCGCCTAAAATCATACCA AAATAAAAAGAGTGTCTAGAAGGGTCAT ATAACCGGTGCATGCGCTAGC
BAR1.61.2	60 nt homology upstream of <i>BAR1</i> control sequences (-338 to -279) + UP	ACATTGTGTCATTTATTATATTAGAGATG CGTTGTCCCTGTTTTCTACCTCCGACATC ACCGGTGCATGCGCTAGC
BAR1.61.3	Reverse complement of 60 nt homology from <i>BAR1</i> ATG to +60 of ORF + DN	GTTAATAATCGCGAAACTCGCCAAAATAA GTTTCAAACAAAGATGATTAATTGCAGAC ATGACGCTCTGTACACCGCGG
URA3.for	Forward primer for split- <i>URA3</i> PCR, IpO plasmids	CACAGTTAAGCCGCTAAAGGC
URA3.rev	Reverse primer for split- <i>URA3</i>	AGTATATTCTCCAGTAGCTAGGGAGCC

Name	Purpose	Sequence (5' to 3')
	PCR, IpO plasmids	

U2 and D2, single underline. UP and DN, double underline. Restriction sites, italics.