Oligonucleotide-Mediated, PCR-Independent Cloning by Homologous Recombination

BioTechniques 30:520-523 (March 2001)

ABSTRACT

We have developed an oligonucleotide-mediated cloning technique based on homologous recombination in Saccharomyces cerevisiae that allows precise DNA sequences to be transferred independent of restriction enzymes and PCR. In this procedure, linear DNA sequences are targeted to a chosen site in a yeast vector by DNA linkers, which consist of two partially overlapping oligonucleotides. The linkers contain relatively short regions of both yeast vector sequences and insert sequences, which simulate homologous recombination between the vector and the insert. The linkers can also contain sequences not found in either the vector or the insert (e.g., sequences that encode ribosome binding sites, epitope tags, preferred codons, etc.), thus allowing modification of the transferred DNA. Linkers can be designed such that DNA sequences can be transferred with just two reusable universal oligonucleotides and two gene-specific oligonucleotides. This cloning method, which is performed by co-transforming yeast with linear vector, substrate DNA, and unannealed oligonucleotides, has been termed the yeast-based, oligonucleotide-mediated gap-repair technique (YOGRT).

INTRODUCTION

To study a given gene or gene product, researchers often need to transfer precise DNA sequences from one vector to another, a process that has been made simpler by the advent of PCR. Although PCR-based cloning has been the workhorse of gene cloning for the last decade, it nevertheless has its drawbacks. For instance, some DNA sequences are simply recalcitrant to PCR, thereby requiring multiple experiments to identify appropriate reaction conditions and primers. Because PCR products are usually ligated into a restriction enzyme-digested vector, the ligation is dependent on the availability of restriction sites that are present in the vector but absent in the sequences to be cloned. Additionally, it is sometimes difficult to obtain relatively large, error-free PCR products. Consequently, researchers who require faithful replication of the DNA sequences of interest must ultimately perform DNA sequence analysis on the entire length of each cloned PCR product.

To avoid some of these pitfalls, gap repair cloning techniques (5-9, 12) have been employed. These cloning methods utilize homologous recombination in yeast to join homologous DNA sequences. A recent modification of gap repair (13) describes a method that uses PCR-generated dsDNA linkers to stimulate recombination between a yeast vector and insert DNA. Like most gap repair methods, this technique allows the transfer of DNA fragments independent of restriction enzymes, but requires minimal PCR. The modification described here eliminates PCR altogether and demonstrates that unpurified, single-stranded oligonucleotides can function as recombination linkers.

We have named this cloning method the yeast-based, oligonucleotide-mediated, gap-repair technique (YOGRT).

MATERIALS AND METHODS

Strains, Growth Conditions, and Plasmids

The yeast strain used in this study was YEF473 MATaα his3-Δ200/ his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52 (3). Yeast media have been described previously (2). A high-copy, histidine-selectable, yeast shuttle vector pRS423 (4) and the cytomegalovirus (CMV) promoter-containing pCDN (1) were used to create the yeast-based mammalian expression vector p423CDN (data not shown). To create p423CDN-HAU REB1, p423CDN was restricted with EcoRV, and pUREB1 was restricted with EcoRI. Both were purified on a QIAGEN® PCR purification column (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. One-tenth microgram of EcoRV-digested

Received 19 June 2000; accepted 19 October 2000.

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pRS423CDN (vector), 2 μg EcoRI-digested pUREB1 (insert), and 1 μg each of four oligonucleotides (Table 1 and Figure 1, A and B), or 20 μL of the linker synthesis reaction (unpurified; see below) were simultaneously introduced into YEF473 by the lithium acetate yeast transformation procedure essentially as described previously (6). To select for transformants that contain recircularized plasmids, cells were spread onto two synthetic complete (SC)-His plates. The transformants were pooled by adding approximately 1 mL water to a single plate and scraping the plate with a sterilized glass slide. The cell mixture was poured into an Eppendorf® tube and microcentrifuged at 18000 × g for 30 s. Plasmid DNA was released from the cells as previously described (7). Plasmids were introduced into E. coli strain DH10B® (Life Technologies, Rockville, MD, USA) via electroporation and isolated from E. coli using the QIAquick® mini-prep procedure (Qiagen).

Oligonucleotides and Synthesis of Double-Stranded Linkers

Oligonucleotides (Table 1) were synthesized on a model 394 or 3948 RNA/DNA Synthesizer (Applied Biosystems, Foster City, CA, USA). Double-stranded linkers were created by adding 1 μg each of two partially overlapping oligonucleotides (Table 1 and Figure 1, A and B) to a thin-walled PCR tube that contained 1× Expand™ polymerase buffer (Roche Molecular Biochemicals, Indianapolis, IN, USA), 0.2 mM dNTPs, and 2.5 U Expand polymerase in a final volume of 100 μL. The mixture was cycled 10 times at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Three microliters of each reaction were analyzed on a 2.5% agarose gel. Sequence analysis was performed on an ABI 377 DNA Sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

Subcloning and Modification of a Human cDNA Using Synthetic Oligonucleotide Linkers

To determine if a cDNA clone could be modified and simultaneously subcloned into a mammalian expression vector using oligonucleotides as linkers, we chose the human UREB1 cDNA (GenBank® accession no. AF057569), which encodes a protein involved in ubiquitination, as a test gene. Two pairs of oligonucleotides (Table 1 and Figure 1, A and B) that contain sequences found in both the yeast vector and the insert sequences were designed. The oligonucleotide pair used to modify and target the 5’ end of UREB1 to the promoter region of p423CDN consisted of two partially overlapping oligonucleotides. The first oligonucleotide (Table 1, oligonucleotide 1) was a vector-specific 71-mer that contained a portion of the p423CDN CMV promoter and several nucleotides downstream of the promoter. Its partner oligonucleotide (Table 1, oligonucleotide 2) was a 105-mer that contained, from 5’ to 3’, the reverse complement of UREB1 (nucleotides 4–43), 27 nucleotides that encode an HA tag (YPYDVPDYA), a six-nucleotide ribosome binding site (8), and the 3’ 25 nucleotides of the vector-specific oligonucleotide.

The oligonucleotide pair used to target the 3’ end of UREB1 to p423CDN also consisted of two partially overlapping oligonucleotides. One oligonucleotide was a vector-specific 75-mer (Table 1, oligonucleotide 4) that contained the reverse complement of sequences immediately upstream of the transcription terminator sequences of p423CDN. Its partner oligonucleotide (Table 1, oligonucleotide 3) contained the 3’ end of UREB1 (nucleotides 994–1044) and 25 bases that complement the 3’ end of the vector-specific oligonucleotide. Both oligonucleotide pairs contain a 25-nucleotide overlap and 5’ overhangs.

To clone UREB1 into p423CDN, cells of strain YEF473 were cotransformed with EcoRV-digested p423CDN, EcoRI-digested pUREB1, and either the unpurified oligonucleotides described above or the unpurified double-stranded linkers (see Materials and Methods). (It is worth noting that the UREB1-containing EcoRI fragment was not purified from the vector sequences and that the UREB1 cDNA was flanked by 491 bp on one side and by several thousand base pairs on the other.) Both transformations yielded approximately 10,000 transformants...
transformants. Approximately half of
the transformants from each was
pooled, and their plasmids were res-
cued to E. coli. Restriction analysis re-
vealed that 7 of 17 plasmids from the
oligonucleotide-containing transfor-
mation contained the modified UREB1 se-
quence inserted at the appropriate loca-
tion, compared to 13 of 18 plasmids
from the double-stranded linker trans-
formation. Sequence analysis was per-
formed from approximately 200 bp
upstream to approximately 200 bp
downstream of the recombination junc-
tions on four UREB1-containing plas-
mids from each of the transformations.
In each case, two of the plasmids con-
tained the desired sequence and two
contained single-nucleotide deletions in
the regions corresponding to the oligo-
nucleotides. [Note that, despite per-
forming more than a dozen subclonings
by YOGRT and sequencing more than
40 candidate clones, mutations have
been identified only in sequences that
correspond to the oligonucleotides.
These mutations could be caused by
oligonucleotides that have become oxi-
dized by dimethyl sulfoxide (DMSO) in
the transformation reaction or could be
the result of imperfect recombination.]
The negative control, YEF473, trans-
formed simultaneously with only
EcoRV-digested p423CDN and EcoRI-
digested pUREB1, yielded several hun-
dred yeast transformants. Of the 18
plasmids analyzed by restriction analy-
ysis from these transformants, none
showed the desired restriction pattern.

Taken together, these results show
that both partially overlapping oligonu-
cleotide pairs and double-stranded link-
ers can efficiently stimulate homolo-
gous recombination between linear
extrachromosomal DNA fragments in
yeast. These results also show that par-
tially overlapping oligonucleotide pairs
appear to stimulate homologous recom-
bination approximately half as effi-
ciently as the double-stranded linkers.
This result is typical of the approxi-
mately two dozen subclonings we have
performed by YOGRT and by double-
stranded linker gap repair, in which we
have found that YOGRT usually pro-
duces a cloning efficiency of 25%–
50%, whereas double-stranded linkers,
synthesized as described here, routine-
ly produce cloning efficiencies of

Table 1. Oligonucleotides Used to Create Linkers

<table>
<thead>
<tr>
<th>Number</th>
<th>Length (nucleotides)</th>
<th>Sequence*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>71</td>
<td>GGAGGTCTATATAAGCAGAGCTGGGTACGTGAACGTACC</td>
</tr>
<tr>
<td>2</td>
<td>105</td>
<td>ACAAGGCGTACATAGGGTTAAACATCTCTCGAGAGTATAGGTCGTAGATCAGTGTGGTCCTGGAGACGCCATCGAATTCGGTCATCAG</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>GCTACTGTTGCTATCCAGAGGTGCTCTGAAGGCTTTGGGCTGGCCTAATAGGTATATCTACGTATGATCAGCCTCCG</td>
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<tr>
<td>4</td>
<td>75</td>
<td>GCCACCGGGGAGGGCCAACAAACAGATGGGTCGGACACTAGAAGGCACAGTGGACGAGGCTGATCATACGAGGCTGATCATACG</td>
</tr>
</tbody>
</table>

*Sequences are written 5’ to 3’.

Figure 1. Diagram of oligonucleotide pairs and potential mechanism for oligonucleotide-mediated recombination. (A) Design of the oligonucleotide pairs. The diagram represents the oligonucleotides used to stimulate recombination between the vector sequences and UREB1. Numbers in parentheses refer to the oligonucleotide numbers (Table 1). (B) A model for oligonucleotide-mediated recombination. The single-stranded oligonucleotides anneal to create partially double-stranded linkers. Crossover occurs between identical sequences in the vector and oligonucleotides 1 and 4 and between identical sequences in UREB1 and oligonucleotides 2 and 3, thus creating the p423CDNUREB1. We point out that this model is merely speculative and that many other possible mechanisms exist.
60%–80%. Sequence analysis of candidate clones from these experiments revealed that approximately 80% of the junctions contain the correct sequences. [Note that preannealing the oligonucleotides before transformation did not improve the cloning efficiency (data not shown).]

The use of single-stranded oligonucleotides to transfer precise DNA sequences offers several advantages compared to other cloning methods. (i) Relatively large sequences can be transferred and modified without fortuitous restriction enzyme sites or PCR. (To date, the largest fragment transferred by YOGRT was 6.4 kb.) (unpublished results). (ii) Potential problems encountered with PCR (e.g., substrate length, GC content, primer annealing, PCR conditions, etc.) are eliminated. (iii) Oligonucleotides designed to contain only vector sequences can be reused for cloning many DNA sequences into a given vector. (iv) The DNA transfer is non-labor intensive. Nevertheless, synthesis of double-stranded linkers as described here is a relatively simple procedure that can enhance the cloning efficiency. In fact, the synthesis reaction need not be purified before transformation nor subjected to gel analysis because a failed synthesis reaction simply means that the subsequent recombination events will be stimulated by oligonucleotides rather than completely double-stranded linkers. We have not determined the minimal length of single-stranded overhangs or the minimal length of overlap for an oligonucleotide pair, which is required for efficient recombination. However, as is true for ds-DNAs (11,13), we have found that 40 nucleotides of overlap efficiently stimulate recombination.

REFERENCES


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Received 7 August 2000; accepted 27 October 2000.

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