Instability of a Multiple Copy Enhancer in Plasmid Vectors: Practical Considerations

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The insertion of multiple copies of DNA elements into plasmid vectors is frequently used to amplify the functions of the element. For example, tandem repeat elements can generate a hyperactive bait in yeast one-hybrid systems (3), and multiple copies of enhancer elements can greatly increase promoter strength (8). The latter effect may be important in vector development for transgenic animals and to amplify gene expression in gene therapy. The former effect makes it of direct practical significance in situations involving the cloning of multiple copy short element repeats.

A construct containing four tandem copies of a 60-mer (consisting of the erythropoietin hypoxia-inducible enhancer element with linker cloning sequences) was generated by standard molecular techniques. Briefly, an oligonucleotide containing the sequence 5'-GATCCGGCCCTACGTGCTGTCTCACACAGCCTGTCTGACCTCTCGACCTACCGGCCG-3' with BamHI restriction ends and 5' phosphorylation was synthesized (Life Technologies, Gaithersburg, MD, USA), polyacrylamide gel electrophoresis (PAGE)-purified, annealed at equimolar concentrations and cloned into BamHI-cut pGEM®-4Z (Promega, Madison, WI, USA) at a 50:1 (insert:vector) molar ratio using a Fast-Link™ DNA Ligation Kit (Epitcentre Technologies, Madison, WI, USA). Recombinants were selected with blue/white screening and verified by restriction digestion. Individual clones were amplified in DH5α™ E. coli (Life Technologies), purified using anion-exchange columns (Qiagen, Valencia, CA, USA) and sequenced. A clone containing all four copies in the sense orientation was selected. This construct was digested with EcoRI and HindIII (New England Biolabs, Beverly, MA, USA) to excise a 291-bp fragment, which was purified twice with the QIAquick™ Gel Extraction Kit (Qiagen) by electrophoresis through 2% agarose. The purified product, which displays no evidence of contaminating DNA (Figure 1a), was ligated into the following EcoRI/HindIII-digested vectors: (i) pGEM-4Z; (ii) pBluescript® SK(-) (Stratagene, La Jolla, CA, USA); (iii) pSP72 (Promega); and (iv) pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), at a 3:1 (insert:vector) molar ratio. Positive recombinant clones were amplified in DH5α E. coli, purified and digested again with EcoRI and HindIII.

As shown in Figure 1b, all of the plasmid preparations contained mixtures of constructs with varying copy numbers of the repeat, even after a single overnight growth from a transformant colony to stationary phase. Deletions are evident in all vectors, and amplifications to 5X and 6X are apparent in pSP72.4X, pBS.4X and pcDNA3.1.4X and to 5X in pGEM-4Z.4X. The differing proportions of copy number present in each vector demonstrate the varying instability of each insert/vector combination; these results are reproducible in other transformants similarly grown and analyzed. Plasmids grown and purified from frozen glycerol stocks prepared from these initial constructs showed similar digest patterns in three subsequent preparations over the course of two months (data not shown).

To test for E. coli-strain dependence, we transformed pBS.4X into three different E. coli strains: DH5α, endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 U196 deoR; JM109, endA1 hsdR17 supE44 thi recA1 gyrA96 relA1 e14 McrA (Promega) and GM2163, ara-14 leuB6 thi-1 fluA31 tsx-78 galK2 galfT2 supE44 hisG4 rpsL136 xyl-5 mtl-1 dam13::Tn9(Cam') dcm-6 merB1 hsdR2 merA (New England Biolabs). DH5α and JM109 are deficient in recombination (recA) and in restriction (hsdR). GM2163 contains recA and is recombination-proficient but is deficient in restriction/methylation (hsd, mcrA, mcrB1, dcm and dam). Nine colonies were randomly selected and amplified in mini-cultures, and plasmid DNA was purified by alkaline lysis. EcoRI/HindIII digests (Figure 2) reveal the high-frequency deletion or amplification of the four-copy repeat. DH5α displays the most integrity, maintaining four-copy repeats as the major band in seven colonies, amplification to 5X in one colony and deletion to 3X in another. JM109 maintains four-copy repeats as the major band in five colonies, one...
deletion to 3×, two deletions to 2× and one deletion to 1×. GM2163 displays the least fidelity, maintaining four-copy repeats as the primary band in three colonies, four deletions to 2× and three deletions to 1×. As mentioned in the figure legend, although the major band may be four (or more or less copies), minor bands corresponding to other copy numbers are present in each sample lane. The results support previous reports that these deletion/amplification products occur independently of recA, confirm the extremely high-frequency rearrangements of this 4× repeat and suggest significant differences among E. coli strains in recombination frequency. These results were confirmed in three separate experiments with pBS.4X and these E. coli strains (Figure 2 shows a representative result of copy-number recombination) as well as with another 72-bp repeat element with an unrelated sequence (data not shown).

Finally, we subcloned the four-copy repeat from pGEM-4Z.4X directly into the SmaI site of the pGL2-Basic Vector (Promega) and inserted a minimal promoter to create pGL2αMHC-86 (see Reference 7 for cloning procedures). This was subjected to successive overnight growths in JM109 and analyzed by XbaI (New England Biolabs) restriction digestion and sequencing. As Figure 3 shows, there were no apparent rearrangements of the same repeat elements in these preparations, indicating significantly increased stability compared with the vectors described in Figure 1. We cannot rule out the possibility of the lower-frequency rearrangements similar to those described previously for 2-copy repeats (2,5). We also transformed both DH5α and GM2163 with pGL2αMHC-86 and found a copy number modification

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**Table 1. Properties of Vectors and Insert**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size(bp)</th>
<th>Origin</th>
<th>Position (bp)</th>
<th>Uni-/Bidirectional Direction to Insert (bp)</th>
<th>Insert Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-4Z.4X</td>
<td>2986</td>
<td>pMB1</td>
<td>509</td>
<td>unidirectional</td>
<td>481</td>
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<tr>
<td>pBluescript SK-.4X</td>
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<td>ColE1</td>
<td>1215</td>
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<td>530</td>
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<tr>
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<td>ColE1</td>
<td>377</td>
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<tr>
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<td>pMB1</td>
<td>3692</td>
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<td>2770</td>
</tr>
<tr>
<td>pGL2αMHC-86</td>
<td>5854</td>
<td>ColE1</td>
<td>3056</td>
<td>unidirectional</td>
<td>3060</td>
</tr>
</tbody>
</table>

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**Figure 1. Detection of recombination products from four-copy repeat plasmid constructs.** (A) A pure 291-bp EcoRI/HindIII fragment was generated by excision from pGEM-4Z (as described in the text) and twice purified through 2% agarose. (B) The 291-bp fragment is ligated into routine subcloning vectors and propagated in DH5α. The purified plasmid is digested to completion with EcoRI/HindIII and resolved on 10% PAGE (TBE). The copy number and corresponding size is indicated to the right.
frequency of less than one in twenty in both strains and no evidence of modified copy number minor banding, again verifying the increased and unique integrity of this insert/vector combination (data not shown). While we do not know the molecular mechanism for the enhanced stability of the 4x repeat in the pGL2 construct, a survey of vector and insert characteristics (see Table 1) reveals that no distinguishing property can account for the inherent stability of pGL2xMHC-86. These data serve to emphasize the diverse functional attributes of the different plasmids used in this study and the importance of both selecting the vector with highest fidelity and routine verification of copy-number maintenance.

The replication slippage model for RecA-independent recombination events of the type described here is widely accepted (6,9). In this model, a stalled replication fork and improper alignment result in the deletion or amplification of direct repeats. It has been determined that the frequency of these events can be minimized by (i) inserting large intervening sequences (1), (ii) deleting particular regions of the vector backbone (2), (iii) moving the repeat region further away from the origin of replication (1) or (iv) changing a base in the repeats every 20 bp (6). Our studies support the multifactorial nature of this recombination and further demonstrate that such events can occur at extremely high frequency. These observations suggest the need for regular inspection of multiple tandem repeats in plasmid constructs after E. coli propagation to ensure that the functional phenotype remains uncompromised by undetected recombination events.

REFERENCES


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