Ligation independent cloning irrespective of restriction site compatibility

Chuan Li* and Ronald M. Evans

Gene Expression Laboratory, The Salk Institute for Biological Studies, Howard Hughes Medical Institute, 10010 North Torrey Pines Road, La Jolla, CA 92186, USA

Received July 15, 1997; Revised and Accepted August 18, 1997

ABSTRACT

Here we report the use of exonuclease to expose complementary DNA between an insert and vector such that annealing becomes independent of restriction site compatibility. We demonstrate that unusual and, in some cases, previously impossible cloning strategies can be readily and efficiently achieved as long as the flanking sequences of the linear vectors are highly related. Furthermore, we show that the bacterial repair system resolves the residual mismatches, overhangs or gaps in a predictable fashion to generate excisable inserts. This approach facilitates cloning regardless of restriction site compatibility and overcomes an important limitation in current cloning techniques.

Conventional cloning requires a DNA insert and a recipient vector to possess either blunt or compatible cohesive ends by restriction digestion. Recently, the exonuclease activity of T4 DNA polymerase or exonuclease III itself has been used to generate short, 2 or 4 nt, cohesive ends similar to those produced by restriction enzyme digestion (1,2). As with restriction enzyme mediated cloning, both strategies still require a ligation reaction. However, it has also been found that by generating longer cohesive ends, the annealed DNA complex becomes more stable and the ligation reaction can be omitted (3–5). This technique is therefore known as ligation independent cloning (LIC).

T4 DNA polymerase and exonuclease III are incompletely processive exonucleases (6–8). This property of incomplete processivity leads to our hypothesis that (i) the gaps and overhangs exist in the annealed complexes of LIC; (ii) once the annealed DNA complex is introduced into the cell, the non-complementary regions (gaps and overhangs) can be effectively repaired by the bacteria. This hypothesis is illustrated schematically in Figure 1.

During the process of making double-cistronic expression vector, it was convenient for us to clone a PCR product designed for one restriction site into an immediate adjacent site without further change of our design by using LIC. The cloning efficiency was estimated by measuring both the transformation efficiency and percentage of positive colonies (PPC). Transformation efficiency was defined by the colony forming unit (CFU) per microgram insert DNA, while the PPC was estimated by restriction digestion of plasmid miniprep DNA from randomly picked colonies. As expected, all six attempted

*To whom correspondence should be addressed. Tel: +1 619 453 4100 x1550; Fax: +1 619 455 1349; Email: cli@axp1.salk.edu
Figure 2. (a) Experimental design. Annealing pattern of insert designed for EcoRI-annealed with SmaI linearized vector. The gaps, overhangs and mismatches are indicated. (b) Cloning efficiencies of the strategy. Shaded box represents average transformation efficiency obtained from six different experiments using DH5α cells. Open box represents average percentage of positive clones measured from restriction digestion of plasmid miniprep DNA from randomly picked colonies. The transformation efficiency of DH5α cells for supercoiled DNA is \(1 \times 10^6\) CFU/µg. (c) Sequence analysis of the junction that contains non-complementary regions. Mismatches and overhangs at 3' junction of the experimental design were correctly repaired. Note that the originally designed restriction site was restored. * represents those ends generated by exonuclease digestion; they can be a few nucleotides longer or shorter.

constructs were obtained and the double-cistronic expression vector was successfully generated (9). The average transformation efficiency was \(1.52 \times 10^4\) CFU (Fig. 2b, shaded box). The average PPC was acceptable at 24% (Fig. 2b, open box). The slightly lower cloning efficiencies compared to previous reports (3–5) are presumably the results of the non-complementary regions in the annealed vector–insert complexes.

From the experimental design, we knew that there would inevitably be gaps, overhangs and mismatches in the annealed insert–vector complexes (Fig. 2a). Figure 2c shows the sequences of one of the repaired junctions. Mismatches and overhangs due to restriction site switch were correctly repaired according to the annealed DNA strand. This was true at each of the junctions of the non-complementary cloning constructs including gaps. This differs from the previously described mismatch cloning which gives two different progeny plasmids (10). Furthermore, the originally designed restriction site (EcoRI) was restored at both the 5'- and 3'-end of the insert and the actually used restriction site (SmaI) was restored at the 5'-not the 3'-end of the insert (Fig. 2c). The restored restriction sites facilitate future analysis of the constructs. Because our method is based on sequence annealing, alignment is automatic and as expected there were no frameshifts in the resulting plasmids. For the same reason, the orientation of the insert with respect to the vector is determined from the PCR primer design which also prevent multiple insertions. The technique requires complementarity of the insert to flanking regions of the linear vector and is most efficient if the insert is cloned into adjacent restriction sites of the same vector or into different but highly related vectors. This strategy is highly reproducible and reliable. We successfully cloned other 16 different inserts designed for BamHI site into adjacent XhoI linearized vectors by this strategy (data not shown). The method has been successfully used in different Escherichia coli strains (DH5α, XL1blue and HB101). The fact that we can effectively transform low-competency BL21 cells, (11) indicates the broad utility of this strategy (data not shown).

The question remains as to the maximal length of gaps, overhangs and mismatches that can be tolerated and what is the minimum length of the complementary region. We believe that bacteria can repair substantially longer overhangs, gaps and mismatches than we demonstrated here, provided that the complementary sequences are sufficiently strong to carry the insert–vector complexes into the cells. Theoretically, a longer complementary region will generate more stably hybridized insert–vector complexes. Therefore, it should result in higher cloning efficiency. The shortest complementary region we used in the strategies for annealing was 8 nt. However, the shortest limit of the complementary region is currently unknown.

While this technique was developed to facilitate complex cloning and subcloning strategies, it has other applications as well. For example, it should aid in linking together overlapping genomic or cDNA clones to create intact genes or full length cDNA. In addition, the stability of the annealed DNA segments should make this approach useful for direct transformation of eukaryote cells. Accordingly, this technique could be used in studying DNA repair in bacteria, yeast and mammalian systems.

ACKNOWLEDGEMENTS

We thank Drs G.Wahl, B.Blumberg, S.Inoue and J.Schwabe for critical reading of the manuscript and helpful discussions. C.L. is a postdoctoral fellow of Howard Hughes Medical Institute (HHMI). R.M.E. is an Investigator of the HHMI at The Salk Institute for Biological Studies. This work was supported by HHMI, grants GM 26444 and HD 2718 from the National Institute of Health (R.M.E.), the March of Dimes and the Mathers Foundation.

REFERENCES