Cassette mutagenesis shows its strength

from J. H. Richards

The hybrid mutants and mutant families created by cassette mutagenesis may advance our understanding of the relationship between function and structure.

STUDIES of the relationship between molecular structure and biological function have gained impetus from improvements in our ability to make changes, both specific and random, in the genetic material, and to observe the functional manifestations of these changes.

One approach in particular, called cassette mutagenesis[1-5], can create specific mutants or families of mutants that cannot be readily obtained by other methods of mutagenesis. This technique involves removing a stretch of DNA flanked on either end by a restriction site, then inserting a new cassette of DNA in its place. The appropriate restriction sites may be present in the gene originally or they can be introduced by site-directed mutagenesis[1,2,6]. Hence there are few restrictions on the sequence of the inserted DNA: it can be long or short, contain many substitutions or have mixtures of bases at different sites. Four specific examples serve to highlight some of the current applications of cassette mutagenesis.

The first illustration concerns the introduction of five amino acid changes in the "outside" surface of the DNA recognition α-helix of 434 repressor with the corresponding amino acids from the recognition helix of P22 repressor[7]. The 434R and the P22R proteins bind only to their cognate operators, which have the following consensus sequences[8,9] (● indicates a centre of symmetry and ⋆, a non-conserved base):

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A⋆T⋆AAG⋆⋆⋆⋆⋆CTT⋆A⋆T        P22 operator
  ACAA⋆⋆⋆⋆⋆⋆⋆TTGT          434 operator
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To substitute the amino acids of 434R with those present in P22R at a homologous position in the helix-turn-helix characteristic of many DNA binding proteins, a 39 bp duplex was synthesized and introduced into a vector containing the rest of the gene for 434 repressor. The resulting 434R/P22R hybrid showed the binding specificity of P22 repressor as measured both *in vivo* and *in vitro*.

Amino acid sequence homologies and three-dimensional structural similarities suggest an evolutionary kinship between β-lactamases and the D,D-carboxypeptides-transpeptidases that cross-link the bacterial cell wall[10-12]. To test this relationship, one objective has been to change a β-lactamase into an enzyme with detectable D,D-carboxypeptidase activity. We have used cassette mutagenesis to create a chimaeric protein containing a 30 amino acid insert from the *E. coli* enzyme PBP5 (MW 44,500):

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 50                 ⋆          78
-DLNSGKvLaeeqnadvRrdpaSltKmmtsG-
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in place of a 29 amino acid region of RTEM-1β-lactamase (MW 28,500):

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 50                 ⋆          78
-DLNSGKiLes-frpeeRFpmmStfKvllcG-
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where ⋆ denotes the active site serine (Y.H. Chang and J.H.R., in preparation). The mutant protein differs in 7 per cent of its amino acids from the parent β-lactamase and does not confer an antibiotic-resistant phenotype. But the chimaeric mutant has acquired appreciable D,D-carboxypeptidase activity, having about 3 per cent the activity of PBP5 toward diacetyl L-lys-D-ala-D-ala.

An experiment with the CYC1 gene of *Saccharomyces cerevisiae*[13] demonstrates the power of combining cassette mutagenesis with DNA synthesis to produce families of mutants for phenotypic screening. In studying the role of the 5′ ends of mRNA in this gene, a 54-mer double-stranded synthetic oligonucleotide was inserted. During the synthesis of one strand of this duplex, one or two random mutations over a 7 bp region were introduced by using a relative amount of 0.71 M of wild-type base to 0.097 M of each of the other three bases. Primer extension was used to test the effects of these mutations on the site selection of mRNA 5′ ends; a strong preference is apparent for 5′ ends that align with an A residue preceded by a short tract of pyrimidine bases.

The usefulness of coupling cassette mutagenesis with the synthesis of oligonucleotide mixtures is also exemplified by a study of the role of Thr 71 in β-lactamase[14]. This residue is conserved in all class A β-lactamases and lies immediately adjacent to the active site Ser at 70. All 19 possible mutants at this site were created by synthesizing two strands of DNA with essentially equimolar concentrations of A, T, G and C at the first and second, and of G and C at the third, positions of the codon for residue 71.

The resulting duplexes were incorporated into a modification of pBR322 (Fig. 1). From 108 of the resulting colonies, mutants with all 20 amino acids at residue 71 were characterized phenotypically, Surprisingly, cells producing any of 14 of the mutant β-lactamases displayed appreciable resistance to ampicillin; however, the mutants are less stable to cellular proteases than is wild-type enzyme. The results suggest that Thr 71 is not essential for binding or catalysis but plays an important role in the stability of the β-lactamase protein. □

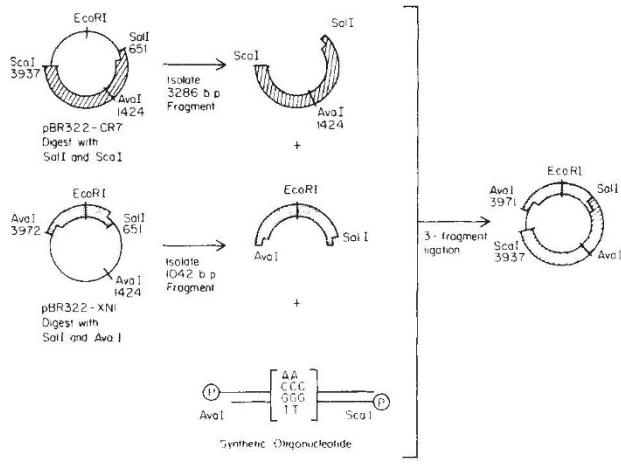

Fig. 1 Design of three-fragment ligation for inserting the mixture of oligonucleotides in pBR322. Bp, base pair. The details of this procedure are typical of most examples of cassette mutagenesis.

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