Gene Therapy (1999) 6, 442–447

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BRIEF COMMUNICATION

Efficient and precise engineering of a 200 kb β-globin human/bacterial artificial chromosome in E. coli DH10B using an inducible homologous recombination system

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Gene therapy studies require techniques that allow alteration of human genomic DNA sequences. Bacterial artificial chromosome cloning systems (BACs/PACs) bridge the gap between vectors with small inserts and yeast artificial chromosomes (YACs). We report the use of a second generation BAC vector, pEBAC, containing eukaryotic selectable markers and combining some of the best features of the BAC, PAC and HAEC systems, into which a 185 kb sequence containing the human β-globin gene cluster was retrofitted. To permit the introduction of mutations corresponding to those causing human pathology, we have adapted an inducible homologous recombination system for use in E. coli DH10B cells, the host strain for BACs and PACs. Using this system, we have introduced PCR fragments carrying a selectable marker and a reporter gene downstream of the IVS I-110 splicing mutation into a specific site within the β-globin gene sequence. The use of this inducible system minimises the risk of unwanted rearrangements by recombination between repetitive elements and allows the introduction of relevant modifications or reporters at any specific sequence within BACs/PACs in E. coli DH10B cells.

Keywords: *homologous recombination; β-globin; PAC; BAC; recE; recT*

PACs[1] and BACs[2] are used increasingly for long-range physical mapping,[3,4] positional cloning of disease genes,[5] whole genome sequencing projects[6] and functional studies.[7,8] However, the lack of convenient techniques for performing genetic manipulations on BACs/PACs in the host *E. coli* DH10B strain imposes serious limitations for functional analysis.

Homologous recombination in an F plasmid-based vector in *E. coli* was first used in 1989 to join overlapping Drosophila cosmid fragments to form a 125 kb fragment.[9] Homologous recombination has also been used recently to introduce targeted modifications in BACs,[10–12] but the available techniques either require the construction of shuttle plasmids or are not directly applicable in DH10B cells.

The bacteriophage λ Red-Gam and the RecE systems promote homologous recombination between linear DNA fragments and circular plasmid molecules or the host chromosome.[13,14] The reaction catalysed by the RecE pathway, termed ET cloning,[14] could be transferred to other *recBC* strains by cloning part of the *recET* operon into the l-arabinose-inducible expression plasmid pBAD24 to give plasmid pBAD24-trecET (YZ and AFS, personal communication). A modified plasmid (pBAD-ETγ) was constructed by Zhang *et al*,[14] with the *gam* gene of bacteriophage λ being used as a natural inhibitor of recBCD nuclease, to allow ET cloning in *recBC*+ strains of *E. coli*. This plasmid enabled targeted modification of a 76 kb Drosophila P1 clone in its *recBC*+ host strain (NS3145) with PCR fragments having about 50 bp of homology in each arm.

A second generation BAC/PAC cloning vector, pEBAC140, (Figure 1a, PAI and J-M Vos), combining features of the first generation PAC[1] and BAC[2] cloning systems and the HAEC system[15] was used in the initial efforts to optimize homologous recombination. EBAC/148β (Figure 1b) contains the entire β-globin locus (about 73 kb) in a 185 kb *Not*I genomic fragment from clone PAC/148β,[16] cloned into the *Not*I site of pEBAC140. A stable human nonerythroid DR cell line was established with EBAC/148β under hygromycin selection, carrying EBAC/148β in episomal format.[17] Analysis of the untransfected and transfected DR cells containing EBAC/148β by RT-PCR, showed that expression of β-globin mRNA was only detectable in the transfected cells (data not shown), indicating that EBAC/148β contains all the endogenous elements required for expression of the genes at the β-globin locus. We wished to introduce modifications to this genomic clone to facilitate functional analysis and gene therapy approaches to thalassaemia.

We obtained precise homologous recombination of PCR fragments of a kanamycin resistance (Km^r) gene

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Received 30 September 1998; accepted 13 January 1999