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Single base, site-directed mutagenesis of a 90 kilobase-pair P1 clone

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Site-directed mutagenesis of cloned DNA has been an invaluable tool in the investigation of the regulation and function of genes and their products. However, all approaches are limited by the size of DNA amenable to such procedures and sub-cloning from and into large DNA can be difficult. This report describes a strategy to introduce a point mutation into a 90 kbp P1 plasmid. We utilized RecA Assisted Restriction Endonuclease (RARE) cleavage (1,2) of two restriction sites surrounding the targeted base within the P1 plasmid to permit removal of a small DNA fragment. This fragment was mutated in an M13 vector and re-inserted into the P1, again utilizing RARE cleavage. To simplify screening of potential clones we found it advantageous to include selectable markers during reinsertion that were easily removed, again with the aid of RARE cleavage. The protocol is outlined in Figure 1.

A 1550 bp EcoRI fragment was deleted from a P1 plasmid that contained a 75 kbp insert encompassing the entire 43 kbp human apolipoprotein B gene (3) (Figure 2, lane 5), using RARE cleavage with oligomers 1 and 2 as previously described (2). Following digestion of the P1 plasmid with EcoRI the restriction enzyme was heat inactivated and the DNA subjected to phenol/chloroform extraction and ethanol precipitation. The P1 plasmid was re-ligated with T4 DNA ligase (New England Biolabs) and electroporated into E.coli strain NS3529 electrocompetent cells. To identify bacterial colonies containing the PI plasmid lacking the 1550 bp EcoRI fragment, colonies

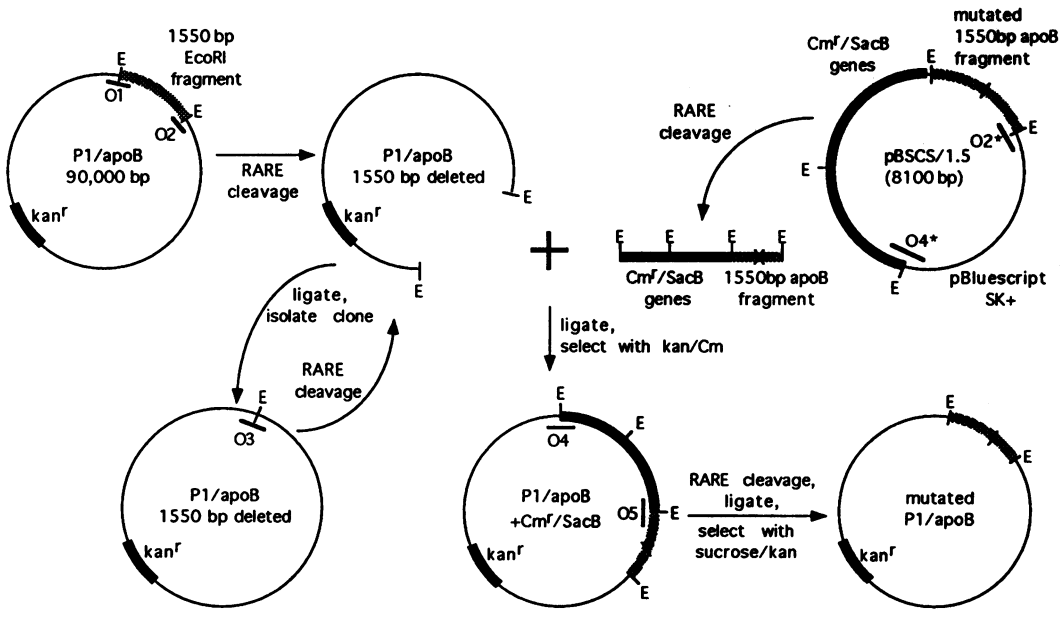

Figure 1. Strategy for removal of a 1550 bp fragment and re-insertion of the mutated fragment into a 90 kbp P1 plasmid. O1, O2, O3, O4 and O5 are synthetic oligomers of 60 bases complementary to the base sequence surrounding the EcoRI sites with the restriction site positioned centrally. O2* and O4* (equivalent to O2 and O4 respectively) are complementary to only one half of the duplex sequence surrounding the EcoRI sites. E; EcoRI site, O; oligomer, Cm^r; chloramphenicol resistance, kan^r; kanamycin resistance.

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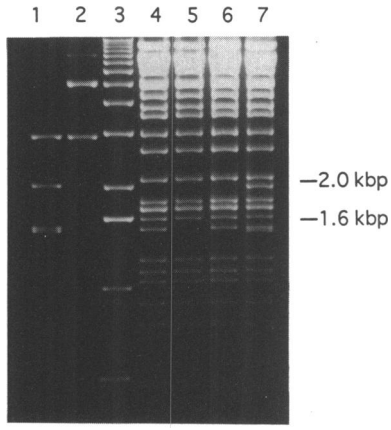

Figure 2. EcoRI restriction enzyme analysis of pBSCS/1.5 (0.17μg) and selected P1/apoB clones (2.5μg) in 0.8% agarose. Lane 1; pBSCS/1.5, lane 2; pBSCS/1.5 with RARE cleavage (as described in Figure 1), lane 3; 1 kb ladder (Gibco BRL), lane 4; P1/apoB, lane 5; P1/apoB with 1550 bp deletion, lane 6; P1/apoB containing mutated 1550 bp fragment re-inserted, lane 7; intermediary P1/apoB clone containing the Cm^r/SacB/1550 bp fragment.

were screened using lack of hybridization to the 1550 bp fragment.

The 1550 bp fragment was gel purified and cloned into M13BM20 (Boehringer Mannheim) and subjected to site-directed mutagenesis by established procedures (4,5) to introduce a single base change (data not shown). For re-insertion of this fragment into the deleted P1 plasmid the chloramphenicol resistance (Cm^r) and SacB genes were introduced along with the mutated 1550 bp fragment to simplify screening. The SacB gene product results in death of the bacterial host when grown in 5% sucrose media so its subsequent removal acts as a selection (6). A DNA fragment containing the Cm^r and SacB genes was cut from pOT2-SacB using a partial ApoI digest and ligated to a fragment obtained by partial EcoRI digestion of pBluescript SK+ (Stratagene) containing the mutated 1550 bp fragment (regenerating the EcoRI sites). The Cm^r/SacB/1550 bp segment (5100 bp) was then removed by RARE cleavage using oligomers 2* and 4* (Figure 2, lane 2). In this case the 60 base oligomers were only complementary with 30 bases on one half of the duplex sequence but were found to be extremely effective in protecting against methylation.

Following RARE cleavage of the deleted PI with oligomer 3 (complementary to the newly formed EcoRI site), the restriction enzyme was heat inactivated and the DNA subjected to phenol/chloroform extraction and ethanol precipitation. The Cm/SacB/1550 fragment was treated with calf intestinal phosphatase to prevent self-ligation and multiple inserts, then gel-isolated. The vector DNA (0.8μg) was ligated with a 5-fold molar excess of the Cm/SacB/1550 fragment using 800 NEB-units of T4 DNA ligase in a volume of 30μl for 1h at room temperature. The reaction was then diluted 10 fold with ligase buffer and incubated for a further 16h at 16°C. The DNA was ethanol precipitated and electroporated into DH1OB cells (Gibco BRL) at 1.6 kV using 0.1 cm cuvettes. Positive colonies were selected on Chloramphenicol (10μg/ml)/Kanamycin (20μg/ml) plates and plasmids were initially screened by mini-prep. Of fifteen Pl plasmids, three appeared normal by a single restriction enzyme digestion. Extensive enzyme analysis of one of the P1 plasmids (data not shown) was then used to confirm its identity (Figure 2, lane 7). The Cm^r/SacB genes were deleted by RARE cleavage with oligomers 4 and 5 as described above and the plasmid (0.8μg) was re-ligated in a volume of 300μl at 16°C for 16h, ethanol precipitated and then electroporated. Positive colonies were selected on 5% sucrose/kanamycin (20μg/ml) and characterized. One of fifty colonies selected was not re-arranged and contained the desired P1 (Figure 2, lane 6).

In summary, for applications such as site-directed mutagenesis, RARE cleavage is an effective technique for subcloning to and from large plasmids. The utilization of selection markers proved to be more rapid and effective than colony hybridization for isolating modified P1 clones and it is forseeable that the use of selection markers could eliminate the need to isolate the deleted, intermediate form of P1.

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