A simple strategy to generate small deletions using *Bal*31 nuclease

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The definition of the functional boundaries of regulatory DNA elements often includes sequential deletion of DNA using controlled digestion with *Bal*31 nuclease [for example, (1, 2)]. To obtain small deletions (< 100 bp) we have devised a simple strategy involving cloning of the target DNA into the *Eco*RI site of the pBR322 plasmid. The hybrid plasmid is linearized at a unique restriction site present at the 3′ end of the target DNA and used as substrate for controlled *Bal*31 digestion. *Bal*31 nuclease will generate deletions of both ends at approximately the same rate (3). Deletion of the vector end larger than 30–50 bp (assuming that the length of insert DNA 3′ of the site used for linearization is no more than 20 bp) will inactivate the tetracycline resistance gene of pBR322. Thus, selection for tetracycline resistant transformants will yield deletions of the target DNA in the order of 100 bp or less. Using this experimental approach we obtained sequential deletions of the target DNA ranging 20 to 100 bp in length.

As a substrate for *Bal*31 limited digestion we used a 283-bp fragment containing *cmp*, the replication enhancer present in the bacterial plasmid pT181 (4). The *cmp* DNA was cloned into the *Sma*I site of the M13-derived MCS (multiple cloning site) region previously inserted into the *Hin*dIII site of the pT181 derivative pGUG103 (4). The *cmp* insert was digested with *Hin*dIII, gel-purified and mixed with EcoRI-digested pBR322 DNA. The incompatible ends were filled-in with Klenow DNA polymerase and dNTPs and blunt-end ligated. Thus, we generated a plasmid containing the *cmp* insert flanked by unique restriction sites (Figure 1, top line). 10 μg of CsCl purified plasmid DNA linearized at the unique *Xba*I site, which is located 3′ of the target DNA in the MCS region, were incubated in 100 μl of *Bal*31 buffer (20 mM Tris-HCl pH 8.0, 0.2M NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 1 mM EDTA) with 0.25 units of *Bal*31 (Boehringer, Mannheim) at 25°C. Under these conditions approximately 10 bp were removed per min from each terminus of the linear substrate. 20 μl aliquots were withdrawn at 30 sec intervals into a tube containing 5 μl 0.1 M EGTA and 5 μl 0.25 M EDTA. Following 2 extractions with phenol-chloroform and precipitation with ethanol, DNA ends were repaired with Klenow DNA polymerase and ligated with T4 DNA ligase. The ligation mixture was cut with *Xba*I (to eliminate DNA non digested with *Bal*31), deproteinized, ethanol precipitated and used to transform *E. coli* AB259. Selection for transformants was on L-agar plates containing 5 μg/ml tetracycline or 50 μg/ml ampicillin. Using the protocol of Kraft *et al.* (5), plasmid DNA from tetracycline resistant transformants was prepared and used for 'T-track' DNA sequencing with Sequenase (United States Biochemical) by the dideoxy chain termination method (6). The primer used for sequencing was the commercially available pBR322 '*Hin*dIII primer' (New England Biolabs).

In a typical experiment, 60% of the transformants were tetracycline resistant. Of these, 87% were deletants, as they yielded plasmid DNA that was resistant to *Xba*I digestion. However, if the ligation mixture was not cut with *Xba*I prior to transformation, only as few as 1/6 to 1/3 of the tetracycline resistant transformants had lost the *Xba*I site. Thus, digestion of the ligation mixture with the restriction enzyme used to linearize the plasmid DNA greatly improves the efficiency of the technique. The results of sequencing ten deletants (Figure 1) demonstrate that the strategy used yields unidirectional sequential deletions from 30 to 130 bp in length in the target DNA. In our experiments, the *Xba*I site was 59 bp away from the *Hin*dIII site of pBR322 (which is located in the promoter region for the *tet* gene). Thus, the rate of exonucleolytic digestion with *Bal*31 in the 'vector arm' was significantly lower than that obtained for the 'target arm'. This is probably due to the difference in GC content of the two regions (30% in the target arm versus 53% in pBR322), for it is known that GC content affects the digestion rate of the enzyme (3).

We are in the process of constructing a pBR322 derivative with the MCS region of pUC18/pUC19 inserted at the *Eco*RI site, which could be used as a cloning vector to obtain *Bal*31-generated small deletions of a target DNA insert following the strategy described in this report. Such a plasmid, which will be available upon request, will facilitate transfer of the deleted DNA fragment to other vectors for functional analysis by using the restriction sites in the MCS region 5′ to the target sequence and the *Cla*I or *Hin*dIII sites of pBR322 on the opposite end.

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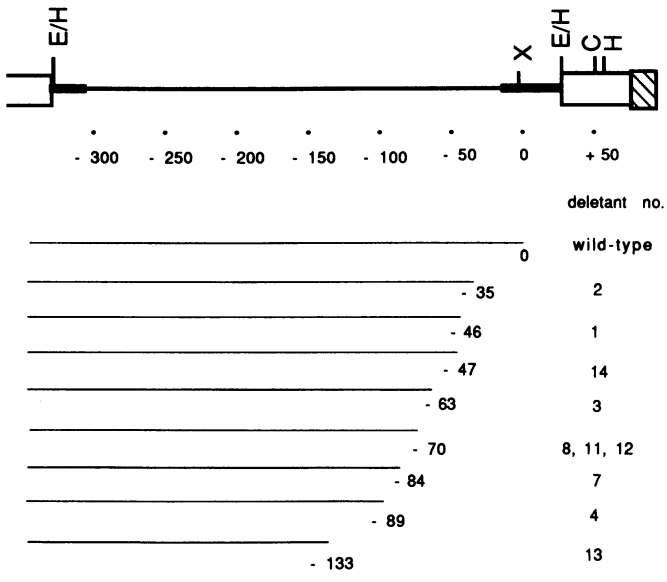

Figure 1. DNA sequence analysis of ten deletion mutants generated in this study. Top. Schematic representation of the plasmid DNA used as substrate of *Bal*31 digestion. The 283-bp *cmp* insert is drawn as a thin line. Solid bars indicate the M13-derived multiple cloning site region. pBR322 DNA is represented by open boxes. The shaded box in pBR322 indicates sequences complementary to the sequencing primer. Restriction sites are indicated as follows: X, *Xba*I; C, *Cla*I; H, *Hin*dIII; E/H, hybrid *Eco*RI-*Hin*dIII site. Bottom. The *Xba*I site used to linearize plasmid DNA is indicated as 0. Negative numbers indicate distance in base pairs from *Xba*I in the 'insert arm'. Positive numbers indicate distance from *Xba*I in the 'vector arm'. Extent of deletion in the 'vector arm' is not shown.