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Insertion of unique sites into YAC arms for rapid physical analysis following YAC transfer into mammalian cells

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The transfer of YACs into mammalian cells has proved to be a valuable approach towards functional analysis of large genetic loci (1). One of the major drawbacks to the use of this technique is the need to verify the integrity of the YAC after insertion into a mammalian chromosome. Such verification usually involves extensive mapping of the YAC before and after transfer, and is especially time-consuming where the DNA is reintroduced into a cell of the same species. To facilitate such analysis we have constructed plasmids which retrofit the vector arms of YACs, allowing insertion of sites for the rare cutter endonuclease I-*Ppo*I along with the neomycin resistance gene which enables selection for the YACs presence following transfer into mammalian cells. I-*Ppo*I recognises a 15 bp sequence which is present in the rDNA of many species but which, according to calculations, should be extremely rare elsewhere (2). Since there is a high probability of the site being absent from YAC inserts, insertion of such sites into YAC arms should allow verification of the integrity of the transferred YAC by a simple digestion followed by hybridisation with a single probe. The plasmids are designed to replace existing selection markers on the YAC arms instead of simply adding new ones. This makes identification of transformants easier, as transformants with the correct phenotype are almost certain to have integrated the plasmid into the YAC arm. Furthermore, the highly effective *URA3* marker gene is absent from the final modified YAC, allowing its use for further modification of the YAC insert where a high efficiency of transformation is required. Figure 1 shows a map of both plasmids and how they integrate into the YAC arms. We have used the plasmids for modification of a YAC (PA-2) containing 460 kb of mouse DNA, which lies in the candidate region for the mouse X inactivation centre and includes the *Xist* gene (3).

Both plasmids described here are pUC19 derivatives (4) and can be propagated in *Escherichia coli* cells, using ampicillin selection. The YAC is retrofitted with the two plasmids sequentially. The first plasmid, pUC-OK, targets the *TRP1* gene on the YAC arm and replaces it by the *LYS2* gene. If the expected integration occurs, the phenotype of the YAC-containing AB1380 cells should change from {Ura+, Trp+, Lys–} to {Ura+, Trp–, Lys+}. pUC-OK is linearised by *Aat*II and *Tth111*I prior to yeast transformation by spheroplasting (5). The transformation efficiency of pUC-OKwas around 400 clones/μg of linearised

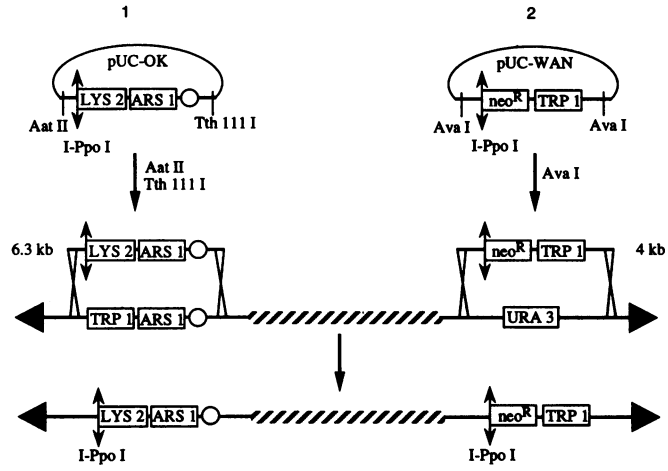

Figure 1. Retrofitting vectors and their recombination into YAC arms. The two bacterial vectors are represented at the top, with the yeast selection markers and the neomycin resistance gene shown as boxes. The open circle represents the yeast centromere *CEN4* and the double arrow represents the I-*Ppo*I site. Sites used for plasmid linearisation prior to yeast transformation are indicated. Sequences for targetting by homologous recombination were derived from the pYAC4 vector for both plasmids. Recombination leading to replacement of markers on YAC arms is shown below, the first event being in the left *TRP* arm and the second event in the right *URA* arm. The sizes of the linearised fragments are indicated and marker genes in the original YAC arms are shown. The YAC insert is represented by the hatched line. The final YAC structure is depicted at the bottom.

plasmid. We obtained six clones with the required phenotype, representing 1% of the Lys+ transformants. Modified YACs were checked by pulsed-field gel electrophoresis (PFGE) and hybridisation to a *LYS2* probe before further modification. All YACs contained the *LYS2* gene. One YAC displayed a size increase of at least 10 kb, but this phenomenon was not characterised any further.

The second plasmid, pUC-WAN, targets the *URA3* gene on the other YAC arm and replaces it with the *TRP1* gene and the neomycin resistance gene under control of the *pgk* promoter (6). The final phenotype of yeast cells should be {Ura–, Trp+, Lys+}. pUC-WAN is linearised by *Ava*I and used to transform a strain containing a YAC already modified by pUC-OK. Transformation

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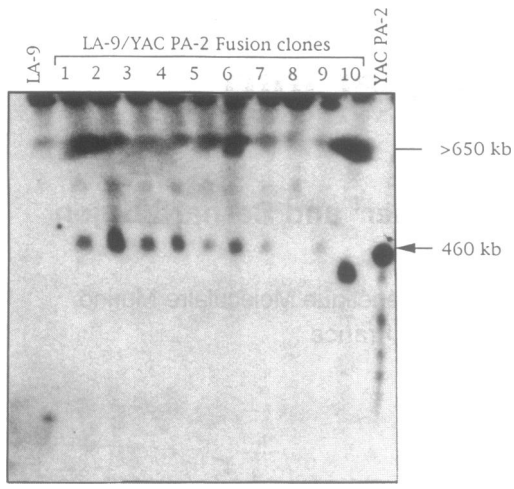

Figure 2. Autoradiogram of I-*Ppo*I digested DNA from mouse L A-9 cells after YAC transfer. DNA from 10 *neo* ^{R} clones (shown 1–10) as well as non-fused L A-9 mouse cells and YAC PA-2 yeast cells was digested with I-*Ppo*I and resolved by PFGE (CHEF apparatus, LKB). The Southern blot of the gel was hybridized with the *Xist* probe (3). The expected position of the 460 kb intact YAC insert is indicated with an arrow. Note the presence of a 460 kb band in eight out of the 10 clones (and absence of this band in L A-9 control cells).

efficiency of pUC-WAN was around 15 clones/μg of linearised plasmid, and 13% of the Trp+ transformants had the required phenotype. Clones with the correct phenotype were checked by PFGE. Two YACs out of the 12 characterised had undergone deletions (~20 and 100 kb long). Hybridisations with the *neo*^{R} and *TRP1* genes after I-*Ppo*I and *Hin*dIII digestions confirmed the expected structure of both YAC arms in YACs which had no visible deletion or insertion.

One of the modified YACs was then transferred into L A-9 mouse cells using the spheroplast fusion technique as previously described (7). Transformants were selected with 600 μg/ml (active concentration) G418 (GIBCO-BRL, Gaithersburg, MD, USA). Ten *neo*^{R} clones were obtained from a fusion involving 10^8 spheroplasts and 2×10^6 L A-9 cells. DNA from these clones was prepared in agarose plugs (3) and digested with I-*Ppo*I (Promega, Madison, WI, USA) using the manufacturer's conditions. The DNA was resolved by PFGE and hybridised with a *Xist* gene probe, which lies within YAC PA-2 (Fig. 2). Eight out of the 10 *neo*^{R} clones can be seen to contain a 460 kb I-*Ppo*I fragment, suggesting that at least one copy of the YAC has been integrated intact into the L A-9 genome. The vectors described here thus permit the rapid assessment, following YAC transfer, of mammalian cell clones or transgenic animals for the presence of an intact YAC and avoid the lengthy physical analysis, involving multiple restriction enzymes and probes, which would otherwise be necessary.

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