Nucleic Acids Research, Vol. 20, No. 12 3135–3138

Mitotic recombination of yeast artificial chromosomes

Jiannis Ragoussis*, John Trowsdale and David Markie[1]
Human Immunogenetics and [1]Cancer Genetics Laboratories, ICRF Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

Received February 25, 1992; Revised and Accepted May 27, 1992

ABSTRACT

Large regions of human DNA can be cloned and mapped in yeast artificial chromosomes (YACs). Overlapping YAC clones can be used in order to reconstruct genomic segments in vivo by meiotic recombination. This is of importance for reconstruction of a long gene or a gene complex. In this work we have taken advantage of yeast protoplast fusion to generate isosexual diploids followed by mitotic crossing-over, and show that it can be an alternative simple strategy for recombining YACs. Integrative transformation of one of the parent strains with the construct pRAN4 (containing the *ADE2* gene) is used to disrupt the *URA3* gene contained within the pYAC4 vector arm, providing the markers required for forcing fusion and detecting recombination. All steps can be carried out within the commonly used AB1380 host strain without the requirement for micromanipulation. The method was applied to YAC clones from the human MHC and resulted in the reconstruction of a 650 kb long single clone containing 18 known genes from the MHC class II region.

INTRODUCTION

The cloning of large (> 100kb) DNA fragments in YACs enables a structural analysis by using a variety of techniques (1). The YACs can be characterised by conventional restriction mapping, fragmentation following homologous recombination at repeat sequences (2) and recombination between two YACs at overlapping regions (3,4). In addition, it has been shown that YAC clones containing human DNA can be successfully transferred into mammalian cells, leading to the expression of the genes contained in the YAC (5). The YAC fragmentation and recombination techniques enable the manipulation of the clones in order to facilitate a detailed functional analysis of large DNA segments following transfer into mammalian cells.

To date YACs have been recombined only in the meiotic phase, which is 100- to 1000-fold more frequent than spontaneous mitotic recombination. However, it is possible to induce mitotic recombination up to meiotic levels by using agents like ultraviolet light (6). If suitable markers are used the reciprocal products of crossing-over can be identified with relative ease (7) thereby avoiding the requirement for micromanipulation. Protocols utilising the sexual cycle for recombining YACs require the appropriate clones to be carried within host strains of opposite mating types. In general this requires transfer of one of the YAC clones to an alternative host either by DNA isolation and retransformation, or by sexual crossing. However, when meiosis is not required for recombination, isosexual diploids produced by protoplast fusion can be utilised, avoiding the necessity for laborious YAC transfer protocols.

The system developed here involves fusion of two overlapping YAC clones, one of which is modified with the pRAN4 construct which contains the *ADE2* gene and disrupts the *URA3* gene contained in pYAC4 (Markie et al. 1992, manuscript in preparation). Recombination was induced by using ultraviolet light and recombinant YAC clones were identified by colour selection.

We demonstrated mitotic recombination in YACs by using overlapping clones containing a large part of the human MHC class II region in order to construct a single clone containing at least 18 genes (8).

MATERIALS AND METHODS

Yeast strains and culture

All YACs were within the host AB1380. Minimal medium was SD (slightly modified from ref. 15) containing 1.7g/l yeast nitrogen base without amino acids and ammonium sulfate, 5.0g/l of ammonium sulfate, 20mg/l arginine, 20mg/l isoleucine, 20mg/l histidine, 60mg/l leucine, 20mg/l lysine, 20mg/l methionine, 50mg/l phenylalanine, 20mg/l valine, 20mg/l tyrosine per liter and 2% glucose. Where appropriate it was supplemented with 20mg/l adenine, 20mg/l uracil and 20mg/l tryptophan. Agar was added to 2% for solid medium. For plating transformation and fusion experiments bottom and top agar was as above but also contained 1M sorbitol.

YAC clones

Two YAC clones were used: 11.2 with a length of 450 kb containing the genes for RING3, DMA, DMB, RING4, RING12, RING9, RING10, RING11, DOB, DQA2, DQB2, DQB3, DQA1, DQB1 and DRB1 (9) and 4D1 with a length of 550 kb containing RING4, RING12, RING9, RING10, RING11, DOB, DQA2, DQB2, DQB3, DQA1, DQB1 and DRB1, DRB2, DRB3 and DRA (10).

* To whom correspondence should be addressed at: UMDS, Division of Medical and Molecular Genetics, Guy's Hospital, London SE1 9RT, UK

Vectors

The pRAN4 modification vector (Markie et al. manuscript in preparation) contains the yeast *ADE2* gene, sequences from pSV2neo and sequences from pYAC4 directing homologous recombination to the right arm of YACs constructed with this vector.

Probes

The probe for *ADE2* was a 2.2 kb fragment derived from plasmid pASZ11 (11), pBR322 was used as a vector probe for pYAC4, RING3, LEE3 and pRTV-I are described in (9), RING4 is described in (12) and RING6 in (13).

Yeast transformation

The transformations were carried out according to Burgers and Percival (14). 1μg of pRAN4 linearised vector was used on 4.5×10^7 4D1 spheroplasts. Transformants were selected on medium lacking adenine and following isolation were individually tested for nutritional requirements. Those auxotrophic for uracil but not requiring tryptophan were considered candidates for targeted recombination and analysed by pulsed field gel elctrophoresis (PFGE) and hybridisation with the ADE2 probe.

Yeast Protoplast Fusion

Yeast were spheroplasted according to Burgers and Percival (14), washed in CaS (1M sorbitol, 10mM CaCl) and equal numbers (5×10^7) of each strain suspended in 100 μl of CaS. 1.5ml of 20% PEG 6000 was added and then treated as for the transformation procedure of Burgers and Percival (14). The cells were plated on medium lacking both adenine and uracil and grown for five days before colonies were isolated and tested by PFGE for the presence of both YACs.

YAC recombination

Mitotic recombination was performed similar to the method described in (15). In particular aliquots of 1000 and 500 cells of the clone 11.2×4D1/20 were each plated out on 82mm plates with medium containing adenine, uracil and tryptophan and exposed to UV light for three seconds. The apparatus used was an Amplirad PCR decontamination chamber (Genetic Research Inc.) containing a 1.8W germicidal lamp (Coast-wave G8T5) with emission at 254nm. The surface of the plates was 14cm from the lamp and measurements suggested a total dose of 30J/m^2 over this exposure time. Survival of the diploid strain used was approximately 60% at this dose. Following incubation for 4 days the plates were screened for the presence of red/white twin spots (colonies with both red and white sectors). Cells from each sector of twin spots were isolated on appropriately supplemented media and then tested for auxotrophic requirements. Potentially recombinant strains were then analysed by PFGE.

RESULTS

Preparation of YACs for recombination

Two overlapping YAC clones, 11.2 and 4D1, were chosen for recombination (Fig.1). They overlap for 350 kb, but are unique for 100 kb on either side, encompassing the DRB and DRA genes towards the telomere, and the RING3, RING6, RING7 genes towards the centromere ((8) and Fig.1).

The YAC clone 4D1 was transformed with BamHI-linearised pRAN4 as described. The transformants analysed contained a chromosome of identical size to the original YAC and

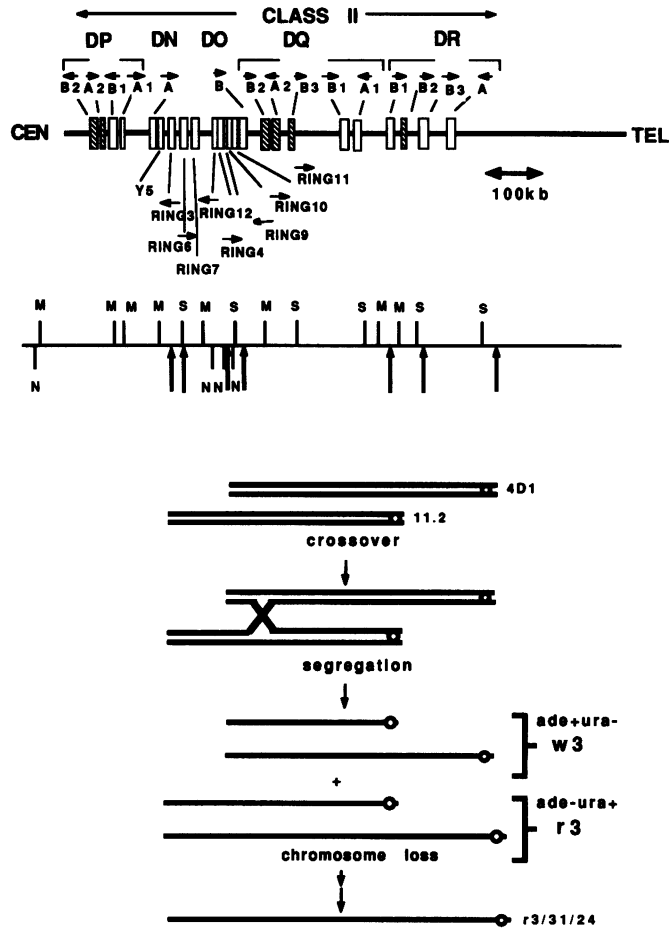

Figure 1. Map of the human MHC class II region showing the position of YAC4D1 and YAC11.2 (Restriction sites shown are for MluI (M), SalI (S) and NotI (N). The crossover event leading to the generation of the recombinant YAC clones, and segregation of chromosomes is shown schematically below taking the Nr.3 twin spot as an example. W indicates the white half of the colony and r the red half. Circles indicate YAC centromeres. r3/31/24 is the 650kb recombinant YAC clone analysed in detail. The restriction map with the enzymes NotI, SalI and MluI is also shown. Arrows indicate the position of the probes used. Tel: chromosome 6 telomere; cen: chromosome 6 centromere.

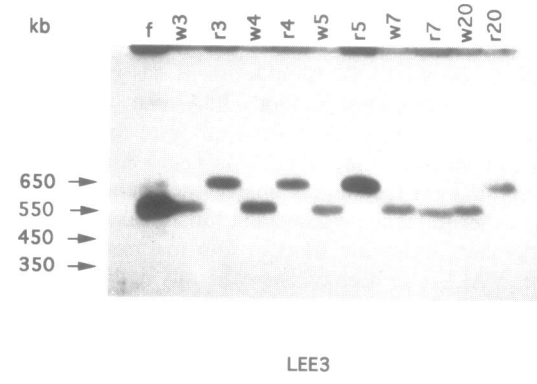

Figure 2. PFGE Southern blot of YAC clone chromosomes derived from the fusion clone 11.2×4D1 (f) and the twin spot isolates 3,5,7 and 20. (w) indicates the white half of the colony and (r) the red half. The blot was hybridised with LEE3, which is a probe 60 kb telomeric of DRA and present on the 4D1 YAC clone (550kb) and the 650 kb recombinant.

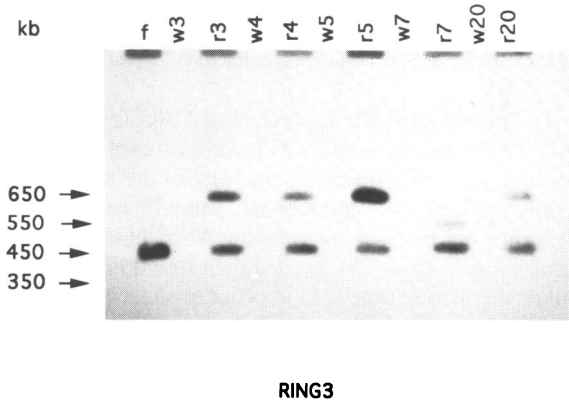

Figure 3. The same blot as in Fig.2 was hybridised with RING3 which is at the end of YAC 11.2 (450kb) and present in the 650 kb recombinant.

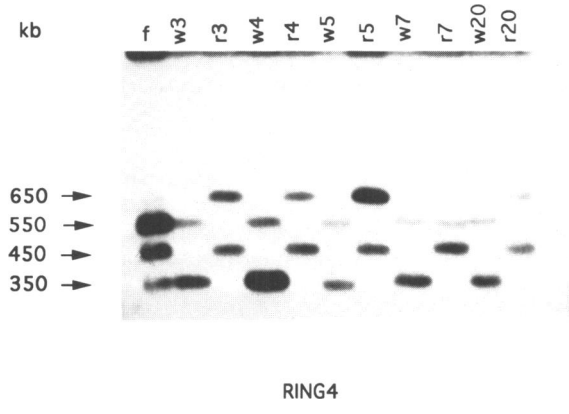

Figure 4. The recombinant YAC clones contain genes from the region of overlap between 11.2 and 4D1. The same blot as in Fig.2 was hybridised with the probe RING4 which is present in both 11.2 and 4D1 and also in the recombinant YAC clones including the 350kb product.

hybridisation confirmed the presence of *ADE2* sequences on this chromosome. One clone (4D1/20) was selected for recombination with the YAC clone 11.2 which was not modified. The two clones were fused as described and one of the resulting isosexual diploids (11.2×4D1/20) containing both YACs was used for the recombination experiment.

YAC recombination and isolation of clones containing recombinants

The recombination experiment was carried out as described in Materials and Methods. Nine (9) putative twin spots were selected from a total of 2000 surviving colonies. Five had the characteristics of a true twin spot ie. the white sector was auxotrophic for uracil and the red sector required adenine. PFGE analysis of four of the twin spots demonstrated chromosomes consistent with the expected products of mitotic crossing-over between 11.2 and 4D1/20 (see Fig.1). Namely the red sector contained a 650kb novel YAC as well as the parental 450kb YAC 11.2 (r3, r4, r5 and r20 Fig.2) and the white sector contained the parental 550kb YAC 4D1/20 as well as a novel 350kb YAC (w3, w4, w5 and w20 Fig2). One twin spot (r7/w7) gave an aberrant pattern, namely the white sector contained a parental

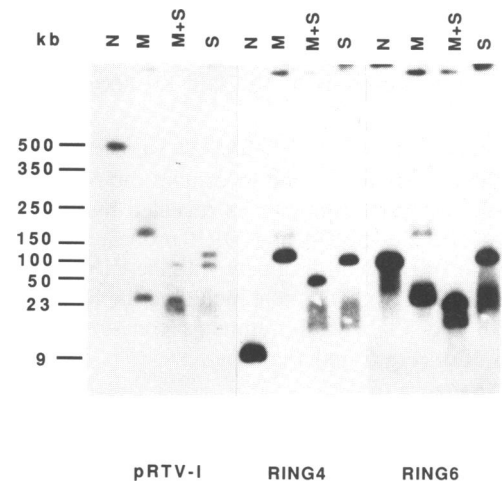

Figure 5. Verification of the recombinant YAC map. PFGE Southern blot of YAC 31/24 DNA digested with NotI (N), MluI (M) and SalI(S). The blot was hybridised with pRTV-I for DRB, RING4 and RING6 probes. The obtained fragments agree with the map in Figure 1.

(550kb) and a recombinant YAC (350kb) as expected where as the red sector contained a parental (450kb) and an unexpected YAC of 550kb which gave a distinct hybridisation pattern from the 550kb parental YAC. This latter YAC is probably the result of a spontaneous deletion of the full length recombinant YAC.

That the recombination product did contain DNA derived from both parental YACs was proven by hybridisations with probes mapping exclusively to the ends of either parent. LEE3 is a probe mapping 60 kb telomeric of DRA and present in the 650 kb YAC and 4D1 exclusively (Fig.2), while RING3 is present in the 650 kb YAC and 11.2 only (Fig.3). Probes in the middle, for example RING4 and DRB (pRTV-I), are present on all YACs as predicted, indicating that the small 350 kb recombination product is derived from the region of overlap between 11.2 and 4D1 (Fig.4 and not shown).

Bands corresponding to all the recombination products discussed above could also be seen, albeit at low intensity, in the primary fusion culture (Fig.4; f).

Isolation of a clone containing only the 650 kb recombinant

Yeast strains isolated following mitotic crossing-over are still diploids and contain two YACs—a recombination product plus one of the parental YACs (see Fig.1). Clones r3 and r5 were chosen for further modification and isolation of the recombinant 650 kb YAC. They were transformed with pRAN4 and the transformants were analysed for the successful integration of pRAN4 within the 650 kb YAC. As expected such clones were obtained along with a set of clones where pRAN4 had recombined with the 350 kb recombination product. The clone r3/31, with pRAN4 targeted to the recombinant 650kb YAC, was selected and grown for 24 hours in YPD medium (15) before plating on medium containing adenine, uracil and tryptophan. Red colonies, presumably due to spontaneous loss of the 650kb YAC, were readily apparent after 3 days, at an approximate frequency of 20%. If loss of each YAC is random then colonies requiring uracil following loss of the parental 11.2 chromosome should be present at the same frequency. 25 white colonies were tested for nutritional requirements and seven requiring uracil were found.

PFGE demonstrated the presence of a single YAC with a length of 650kb in one isolate (r3/31/24) (not shown).

Restriction map analysis of the 650 kb recombinant YAC clone

The restriction sites of clone r3/31/24 were compared to the regional PFGE map and found to contain the correct length of NotI, MluI, and SalI fragments as revealed by probing PFGE blots with RING6, RING4 and DRB probes. For example it contained the 100 kb NotI fragment with the RING6 and RING3 as 11.2 , the 10kb NotI fragment including the RING4 gene and the 190 kb and 35kb MluI fragments containing DRB genes unique to 4D1 (Fig.5 and Fig.1).

DISCUSSION

The human MHC is one of the best characterised regions of the human genome and contains at least 75 genes (8). 20 genes map in the HLA-DNA to HLA-DRA interval of the MHC class II region. The function of some of these genes is not known, while others have related function. For example the RING4 and RING 11 genes are putative peptide transporters, while RING10 and RING12 are proteasome components which may generate peptides for antigen presentation (16, 17, 18).

Functional assays of these regions by introduction of the cloned manipulated DNA into deletion mutants (19) can elucidate the role of multiple genes in antigen presentation. Towards this, the YAC clones have to be tailor made to cover either the entire deleted region or parts of it. We have used YAC recombination to construct YAC variants able to fulfil this task.

The method presented here, an alternative to meiotic recombination, is straightforward and is based on three simple steps. Firstly, modification of one of the YAC clones with pRAN4, secondly, PEG mediated fusion of the two clones and finally, UV induced recombination and colour selection. Another step involving the modification of the recombinant YAC clone followed by loss of the non recombinant YAC, may be desirable for some applications and has been demonstrated. The recombinant YAC clones indicate that mitotic recombination is precise, since three out independent clones contained products of the same size. The recombination had taken place exclusively over the regions of overlap between the two YACs. The restriction enzyme analysis of the recombinant clones revealed that the map agreed fully with the map derived from the original YAC clones and the genomic organisation (8). It is not yet clear what minimum length of overlap is required for succesful recombination and this will be the subject of future studies.

The use of the pRAN4 construct has the advantage that the retrofitted YACs can be introduced directly in mammalian cells by G418 selection (Markie et al. 1992).

The UV treatment used in this protocol carries the risk of introducing point mutations into the YAC. The mutation rate may vary considerably depending on the yeast strain background and the sequences involved. Estimates based upon UV induced reversion with a similar dose in diploid repair proficient yeast at the *cyc1-9* and the *cyc1-131* alleles (approximately 5×10^3 revertants and approximately 2×10^2 revertants/10^8 survivors respectively) (20) suggests point mutation rates between 1 in 40−500 kb. Although it seems likely that most recombined YACs will sustain a number of point mutations during this procedure the possibility of functionally affecting a particular locus is significantly lower. However if the introduction of point mutations into a YAC is of concern the presence of spontaneous recombination at a level detectable by Southern hybridisation (Fig.2;3;4) suggests that recombinants may be isolated without the use of UV.

The strain containing the recombinant YAC is a diploid and further recombination rounds will increase the ploidy status of the resulting clone. The overall simplicity of the method makes it generally applicable for the reconstruction of gene complexes or long genes out of 2−4 overlapping YAC clones. If further rounds of recombination are needed then it will be necessary to manipulate the mating type (21) in order to subsequently reduce the ploidy by sporulation.

REFERENCES

1. Schlessinger, D. Little, R.D., Freije, D., Abidi, F., Zucchi, I., Porta, G., Pilia, G., Nagaraia, R., Johnson, S., Yoon, J.Y., Srivastava, A., Kere, J., Palmieri, G., Ciccodicola, A., Montanaro, V., Romano, G., Casamassimi, A. and D'Urso, M. (1991). *Genomics* **11**, 783−793.
2. Pavan, W.J., Hieter, P., and Reeves, R.H. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 1300−1304.
3. Green, E.D. and Olson, M.V. (1990), *Science* **250**, 94−98.
4. Silverman, G., Green, E., Young, R., Jockel, J., Domer, P., and Korsmeyer, S. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 9913−9917.
5. Pachnis, V., Pevny, L., Rothstein, R., and Constantini, F. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 5109−5113.
6. Roeder, S.G. and Stewart, S.E. (1988). *TIG* **4**, 263−267.
7. Stern, C. (1936) *Genetics* **21**, 625−730.
8. Trowsdale, J., Ragoussis, J. and Campbell, R.D. (1991). *Immunol. Today* **12**, 443−446.
9. Ragoussis, J., Monaco, A., Mockridge, I., Kendall, E., Campbell, R.D. and Trowsdale, J.*Proc. Natl. Acad. Sci. USA* **88**, 3753−3757.
10. Ragoussis, J., Sanseau, P. and Trowsdale, J. (1992). *HLA 91* **Vol.2** (Ed. Sasazuki et al., Oxford Univ. Press) in press.
11. Stotz, A. and Linder, P. (1990). *Gene* **95**, 91−98.
12. Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., and Kelly, A. (1990). *Nature (London)* **348**, 741−744.
13. Kelly, A., Monaco, J., Cho, S., and Trowsdale, J. (1991).*Nature (London)* **353**, 357−359.
14. Burgers, P. and Percival, K. (1987). *Anal. Biochem.* **163**, 391−397.
15. Rose, M.D., Winston, F. and Hieter, P. (1990). *Methods in Yeast Genetics. A Laboratory Course Manual. Cold Spring Harbor Laboratory Press.*
16. Kelly, A., Powis, S., Kerr, L.A., Mockridge, I., Eliott, T., Bastin, J., Uchanska-Ziegler, B., Ziegler, A., Trowsdale, J. and Townsend, A. (1992). *Nature (London)* in press
17. Glynne, R., Powis, S., Beck, S., Kelly, A., Kerr, L.A. and Trowsdale, J. (1991) *Nature (London)* **353**,357−360
18. Kelly, A., Powis, S., Glynne, R., Radley, E., Beck, S. and Trowsdale, J.(1991) *Nature (London)* **353**, 667−668
19. Mellins, E., Kempin, S., Smith, L., Monji, T. and Pious, D. (1991).*J. Exp. Med.* **174**, 1607−1615.
20. Lawrence, C.W and Christensen, R. (1976). *Genetics* **82**, 207−232.
21. Jensen, R. E. and Herskowitz, I. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49**, 97−104.