Recombination during transformation as a source of chimeric mammalian artificial chromosomes in yeast (YACs)

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ABSTRACT

Mammalian DNAs cloned as artificial chromosomes in yeast (YACs) frequently are chimeras formed between noncontiguous DNAs. Using pairs of human and mouse YACs we examined the contribution of recombination during transformation or subsequent mitotic growth to chimeric YAC formation. The DNA from pairs of yeast strains containing homologous or heterologous YACs was transformed into a third strain under conditions typical for the development of YAC libraries. One YAC was selected and the presence of the second was then determined. Co-penetration of large molecules, as deduced from co-transformation of markers identifying the different YACs, was >50%. In approximately half the cells receiving two homologous YACs, the YACs had undergone recombination. Co-transformation depends on recombination since it was reduced nearly 10-fold when the YACs were heterologous. While *mitotic* recombination between homologous YACs is nearly 100-fold higher than for yeast chromosomes, the level is still much lower than observed during transformation. To investigate the role of commonly occurring Alu repeats in chimera formation, spheroplasts were transformed with various human YACs and an unselected DNA fragment containing an Alu at one end and a telomere at the other. When unbroken YACs were used, between 1 and 6% of the selected YACs could incorporate the fragment as compared to 49% when the YACs were broken. We propose that Alu's or other commonly occurring repeats could be an important source of chimeric YACs. Since the frequency of chimeras formed between YACs or a YAC and an Alu-containing fragment was reduced when a *rad52* mutant was the recipient and since intra-YAC deletions are reduced, *rad52* and possibly other recombination-deficient mutants are expected to be useful for YAC library development.

INTRODUCTION

Systems developed in the yeast *Saccharomyces cerevisiae* that are based on artificial chromosome vectors (YACs) have enabled the isolation of large DNAs, up to several megabases that are useful for characterizing the genomes of many organisms (1–11). There are many advantages to using YACs over other cloning systems. For example, they can be manipulated genetically and physically as yeast chromosomes (12, 13). Overlapping YAC clones can be recombined in yeast to construct a single YAC containing a large genomic locus (14–17). Once cloned as a YAC, the DNA can be altered using homologous recombination in yeast. For the case of mammalian systems, the YAC can be subsequently re-introduced into host cells to study the function of the DNA (18–20).

It is essential for the characterization and manipulation of genomic material that the DNA within YACs be accurate, i.e., free of cloning artifacts. However, two major categories of errors—deletions and chimeras—can arise during the development of human YAC libraries. Internal deletions appear to occur during transformation via mechanisms involving recombination (5, 21, 22). Chimeric YACs, which are 20 to 60% of the YACs in human libraries (9, 23–33), could occur by *in vitro* ligation of unrelated DNAs or by recombination *in vivo* (discussed in 26, 34). The relative contribution of ligation versus recombination to YAC chimeras is not known. The large amount of repeated sequences in mammalian DNA, even though diverged, could provide the substrate for recombination. Under conditions limiting ligation, chimeric YACs were frequently observed (34).

In the present work we have developed two model systems to genetically assess the role of recombination in the formation of YAC chimeras. They are based on measuring recombination during transformation between pairs of YACs that are genetically distinguishable or between a YAC and an Alu-telomere DNA fragment when only one of the two molecules is selected. There is frequent co-penetration of large DNA molecules under conditions normally used for the development of YAC libraries. Co-penetration by homologous molecules often leads to

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recombination (i.e., chimeras). The level of transformation-associated recombination is much higher than that observed during the subsequent mitotic growth. The frequency of transformation-associated chimeras can be substantially reduced in a *rad52* recombination-deficient host strain.

MATERIAL AND METHODS

Media and yeast strains

Yeast cells were grown on complete medium (YPD) or synthetic selective medium lacking either uracil, leucine, tryptophan, lysine or histidine, depending on the gene under selection using media described by Sherman, Fink and Hicks (35).

Strain YPH857 (*MATa, leu2-D1, lys2-801, ade2-101, his3-D200, trp1-D1, ura3-52*) was kindly provided by P. Hieter. Strain YPH857-5 is a *MAT***a** derivative of YPH857. A strain with complete deletion of the *RAD52* gene, YPH857-D1 (*MAT***a**, rad52-D2000, *leu2-D1, lys2-801, ade2-101, his3-D200, trp-D1, ura3-52*) was made by one-step gene replacement technique (36) and was described in (22). Two YACs, YAC31 and YAC21 containing 510 kb and 170 kb inserts, respectively, were derived from a YAC library of human chromosome 21 (3). YAC12 is a 360 kb YAC derived from human chromosome 2. This YAC, marked by *URA3* and *TRP1* (YAC12-A) as well as its *LYS2−HIS3* derivative (YAC12-B), has been previously described (37, 22). A 390 kb YAC with a mouse DNA insert, YACYD59, was obtained from P. Hieter (37). Prototrophic markers at the centromere-proximal and -distal ends of YAC21 were modified by gene replacement with the set of marker change plasmids (38). Replacements of markers were verified by TAFE, followed by chromosome blot analysis. The *LYS2−HIS3* derivative of YAC21 was designated as YAC21-B and the parent as YAC21-A (*URA3−TRP1*). Strains carrying pairs of homologous (human:human) YACs or heterologous (human:mouse or human:human) YACs were obtained either by crossing of strains containing different YACs or by retransformation of strains carrying a YAC by a differentially marked YAC. Five different pairs of YACs (Fig. 1) were analyzed for mitotic and transformation-associated recombination: YAC12-A/YAC12-B and YAC21-A/YAC21-B (pairs of homologous YACs), and YAC12-B/YACYD59, YAC12-B/YAC31 and YAC12-B/YAC21-A (pairs of heterologous YACs). The *LEU2*$^+$ strain YVL49 (*MAT***a**, *ura3-52, ade2-101, trp1-D1, lys2-801*) was used as a source of chromosome III in co-transformation experiments. Chromosome fragmentation vectors, pBP108 and pBP109, were used to study recombination between YACs and a linear fragment during co-transformation (39). These *HIS3* marked telomeric vectors contain an Alu human repetitive DNA element. The plasmids having Alu's in opposite orientations were linearized with *Sal*I.

Transformation experiments

Cells were made competent using a high-frequency spheroplast transformation protocol (40) with minor modifications (41, 22). The DNA plugs containing a 1:1 mixture of two different YAC-containing strains were used for transformation. In co-transformation experiments involving the Alu-containing vectors, pBP108 and pBP109, 3 mg of a *Sal*I-linearized YAC fragmentation vectors was added to a plug after agarase treatment.

A 100 ml agarose plug containing about 10^8 molecules of a 360 kb human YAC12 yielded from 50 to 200 Ura$^+$ transformants with the YPH857 recipient strain. Number of YAC molecules per plug was estimated asuming one YAC molecule per cell. A comparable yield of transformants was observed in retransformation experiments involving the 170 kb human YAC21 and with a 340 kb yeast chromosome III. Similar to previous reports (5, 42) the yield of transformants was decreased 2−5 fold for a 510 kb human YAC31. The frequency of transformation with a 360 kb human YAC12 was about 5−10 fold lower when the *rad52* recombination-deficient strain YPH857-D1 was used as a host

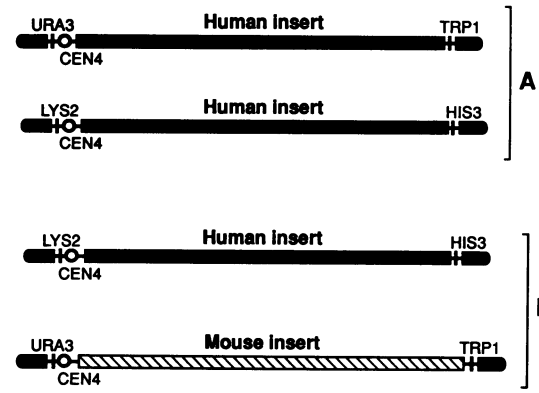

Figure 1. Schematic representation of YAC pairs. Each YAC has a centromere linked marker (*URA3* or *LYS2*) and a distal marker (*TRP1* or *HIS3*). YAC pairs **A** represent two homologous human YACs containing 360 kb and 170 kb human DNA inserts (YAC12-A/YAC12-B and YAC21-A/YAC21-B, respectively). YAC pairs **B** represent two heterologous YACs containing either human/human or human/mouse DNA inserts (YAC12-B/ YAC21-A and YAC12-B/YACYD59, respectively).

Table 1. Co-transformation by an unselected chromosome III or a 360 kb YAC

Molecule selected	Transformants*	Unselected co-transformants*
YAC12-A	1,055	18 (1.7%)**
Chromosome III	1,187	5 (0.4%)

*When the 360 kb YAC12-A was selected (Ura$^+$), the presence of chromosome III (*LEU2* and *MAT***a**) was determined by replica plating. Similarly when the chromosome III is selected (Leu$^+$), the presence of YAC12 (*URA3* and *TRP1*) was determined. Presented are the summarized results of five transformations.
**Numbers in parentheses are the percent of co-transformation events.

Preparation of agarose plugs containing yeast chromosomal size DNAs

For retransformation experiments, yeast cells containing a YAC of interest were cultured in YPD medium to stationary phase. Low-melting-point agarose plugs were prepared at a final density of 10^9 cells per ml containing a 1:1 mixture of two different YAC-containing strains. After purification of the chromosomes and agarase treatment, 100 ml of the sample (equal to 10^8 YAC molecules) was used for spheroplast transformation. Chromosome size yeast DNA for electrophoresis analysis was prepared in a similar way (43). Storage of chromosomal size DNAs was carried out in 0.05 M EDTA, 0.01 M Tris pH 7.5 at 4°C. Transverse Alternating Field Electrophoresis (TAFE) was used for analyzing DNA size (22).

Analysis of YAC co-transformation and inter-YAC recombination

To measure the frequency of co-transformation for chromosome-size DNA, a 1:1 mixture of cells containing two differentially

Table 2. Co-transformation and associated recombination of unselected homologous and heterologous YACs in RAD$^+$ and *rad52* strains

Selected YAC	Unselected YAC	Transformants*	Unselected co-transformants	Recombinants** among co-transformants
RAD$^+$				
Homologous YACs				
360 kb human	360 kb human	2,272	127 (5.6%)	59 (46%)
170 kb human	170 kb human	838	29 (3.5%)	8 (27%)
Heterologous YACs				
360 kb human	170 kb human	811	5 (0.6%)	2 (40%)
360 kb human	510 kb human	870	2 (0.2%)	0
360 kb human	390 kb mouse	819	5 (0.6%)	4 (80%)
rad52				
360 kb human	360 kb human	699	5 (0.7%)	1 (20%)

*Presented are the summarized results for five experiments each for RAD$^+$ and twelve experiments for *rad52*.
**All co-transformants were tested for recombinant YACs (see Materials and Methods). The number in parentheses corresponds to percent of co-transformants that are recombinant.

marked YACs was lysed in agarose plugs and the DNA was used to transform a *ura3* recipient strain. Transformants were selected for the *URA3* centromeric marker of one of the YACs. The transfer of one or both markers of another unselected YAC (*LYS2* or *HIS3*) or two markers of chromosome III (*LEU2* and *MAT*a) was interpreted as a co-transformation event. All YAC co-transformation events were checked for inter-YAC recombination by analyzing the linkage between centromeric and telomeric YAC markers. For this purpose mitotic segregants having lost the *URA3* or *LYS2* marked YACs were selected on 5-fluoroorotic acid or a-aminoadipate plates (44, 45) and the remaining markers were scored.

The rates of mitotic recombination between differently marked homologous YACs were determined by measuring the frequency of homozygotization of the YAC telomeric markers, *TRP1* or *HIS3*. Ura$^+$ Trp$^+$ Lys$^+$ His$^-$ and Ura$^+$ Trp$^-$ Lys$^+$ His$^+$ clones identified during mitotic growth were checked for the linkage of centromeric and telomeric markers. Fluctuation analysis was used to determine the rates of recombination between homologous YACs in recombination proficient and *rad52* strains during mitotic propagation as previously described (22). The linkage between a centromeric marker of one of the YAC (*URA3* or *LYS2*) and a telomeric marker of another YAC was interpreted as inter-YAC recombination event. The rate of loss per generation was estimated using the method of Lea and Coulson (46).

RESULTS

Statement of the problem

Mammalian DNA transformed into yeast cells frequently experiences physical changes such as deletions and mutations that have been proposed to arise by recombination (5, 21, 22). While bimolecular recombination between plasmid molecules has been described (47–49), there have been no detailed studies to determine the efficiency of interactions between chromosomal size DNAs such as YACs containing mammalian DNAs during transformation. To estimate the efficiency of recombination between large molecules during transformation, it is necessary to estimate the efficiency of *co-penetration* of different molecules into the same cell. While co-penetration cannot be measured directly, it can be estimated from the frequency of *co-transformation*. Estimates based on co-transformation (i.e., the coincident incorporation of genetic markers from two different molecules when only one is selected) will depend on the likelihood of establishment of the unselected transforming molecules. In the following experiments we determined the minimum efficiency of co-penetration by large molecules and established that when co-transformation was observed for human YACs, they had frequently undergone transformation-associated recombination.

Co-penetration of chromosomal size molecules and relation to recombination

To estimate the frequency of co-penetration by large molecules, spheroplasts were transformed with DNA from strains containing different YACs. The DNA used for transformation was gently prepared in the same manner used for determining chromosome size. By mixing cells with differentially marked YACs or chromosomes, the ratio of molecules to be examined for co-penetration, co-transformation and recombination could be easily manipulated. Co-transformation was determined for pairs of homologous YACs, heterologous YACs, and for a YAC and the yeast chromosome III. Transformants were selected that had acquired the centromeric marker of one of the YACs or chromosome. Colonies were subsequently examined for the presence of cells containing markers identifying an unselected YAC or chromosome.

Since estimates of co-penetration would depend on the stability of the unselected molecule, we initially examined co-transformation by chromosomal DNA since yeast chromosomes are mitotically stable (compared to YACs). When selection was made for a YAC containing a 360 kb human DNA (YAC12-A), nearly 2% of the transformants had also acquired chromosome III from the transforming DNA. However, when selection was made for the chromosome III, co-transformation was less (Table 1). [Based on TAFE analysis, both molecules were present in all the co-transformants (data not shown)]. These results suggested that opportunities for intermolecular recombination might increase the likelihood of co-transformation. We, therefore, investigated the extent of co-transformation when spheroplasts were transformed with pairs of homologous YACs. Cells containing the differentially marked YACs were mixed and the subsequent chromosome preparations were used for transformation. The average percentages of co-transformation for the 360 kb pair of YACs and for the 170 kb pair of YACs were comparable, 5.6% and 3.5%, respectively (Table 2).

Based on these measurements of co-transformation, we estimated that among transformants selected for a YAC, 2–5% contained an unselected YAC or a chromosome III marker. Since

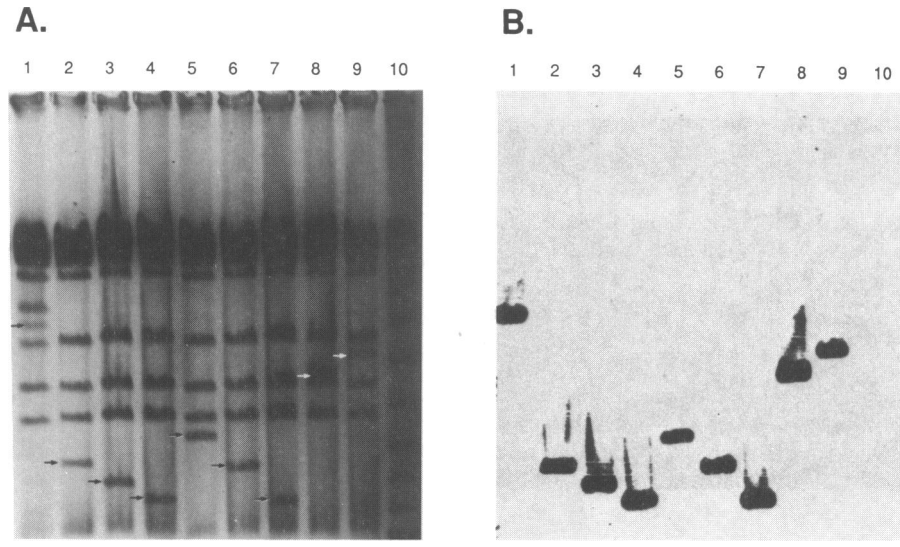

Figure 2. Physical characterization of the products of transformation-associated recombination of a pair of homologous YACs. In **A** the chromosomal size DNAs isolated from clones containing recombinant YACs arisen during co-transformation are ethidium bromide-stained (the picture is a negative rather than a positive). In **B** the YACs are identified with a labelled pBR322 probe. Lane 1: a diploid strain containing two differently marked a 360 kb human YAC12, (YAC12-A and YAC12-B). Lanes 2–9: eight *URA3–HIS3* recombinant YACs arisen during transformation. Note that the sizes of all the recombinant YACs are different from that of the parent YACs. Lane 10: 1 multimers ladder. Arrows indicate positions of the 360 kb original YAC12 and recombinant YACs.

in these experiments the ratio of chromosome III or YAC to all the yeast chromosomes is 1 to 32, it appears that if selection is made for one chromosomal size molecule, there is a high likelihood that another molecule will also penetrate the cell (see Discussion).

If recombination contributed to co-transformation, many of the transformants would be expected to contain molecules that resulted from recombination between the co-penetrating YACs. Formally, these would correspond to chimeric YACs. As shown in Table 2, many of the co-transformed YACs had undergone recombination. Of the co-transformants involving either the 360 kb or the 170 kb pair of YACs, 46% and 27%, respectively, contained single chimeric YACs with markers derived from each of the original YACs. Although the remaining co-transformants contained 2 YACs, they also could have experienced recombination which is not detectable in these experiments. For YAC12, the single chimeric YACs were always shorter than the original. Among 59 YACs analyzed, all were 50–150 kb shorter than the parent (Fig. 2). This differed from the recombinants obtained with the 170 kb pair of YACs in that all the recovered YACs were full-size (data not shown).

Although unlikely, the high frequency of recombination between homologous YACs as well as the high frequency of co-penetration could be explained by an interaction of YACs prior to transformation. Since the plugs containing chromosomal size DNA were melted at 65°C, there might be annealing of AT rich regions between the homologous YACs. This could result in enhanced opportunities for co-penetration and recombination. To test this idea, DNA plugs were prepared in extra low melting agarose (FMC Bio Products). All steps prior to spheroplast transformation were done at temperatures below 55°C. The frequencies of co-transformation and recombination were comparable to those observed when YACs were prepared in the normal manner (data not shown) leading us to conclude that there was no interaction between YACs prior to transformation.

If recombination is important in co-transformation, then the frequency of co-transformation should be reduced for pairs of heterologous YACs. For three pairs of heterologous human YACs, the level of co-transformation, as expected, was approximately 0.5% (Table 2). Since co-penetration is expected to be comparable for heterologous and homologous YACs, we conclude that the reduced opportunities for recombination decreases the likelihood of co-transformation nearly 10-fold. A role for recombination in the establishment of the unselected YAC was supported by the observation that among the co-transformants nearly 1/2 contained a single chimeric YAC as determined genetically and physically (Table 2). The remaining co-transformants contained two molecules.

A yeast *rad52* mutant exhibits reduced levels of mitotic recombination (50–52). If co-transformation involving homologous molecules frequently occurs via recombination, then it should be reduced in a recombination-defective mutant. As shown in Table 2, co-transformation by homologous YACs was about 10-fold decreased in a *rad52* mutant as compared to RAD^+. However, as for the case of heterologous DNAs in RAD^+ strain, co-transformants frequently contained recombined YACs. An examination of co-transformation in a *rad52* mutant by heterologous pairs of YACs was precluded by the lower level of co-transformation and expected a low level of recombination. As discussed below, this could be addressed more quantively using a model YAC and plasmid system.

Co-transformation involving a human YAC and an Alu-containing DNA fragment

Based on the previous results, recombination between YAC DNAs is frequent in co-transformants. Homologous or related sequences such as Alu's that are frequent in the human genome could be sites of recombination. Therefore, we have investigated co-transformation by a YAC and a linearized plasmid pBP108 containing an Alu sequence at one end and a telomere at the other

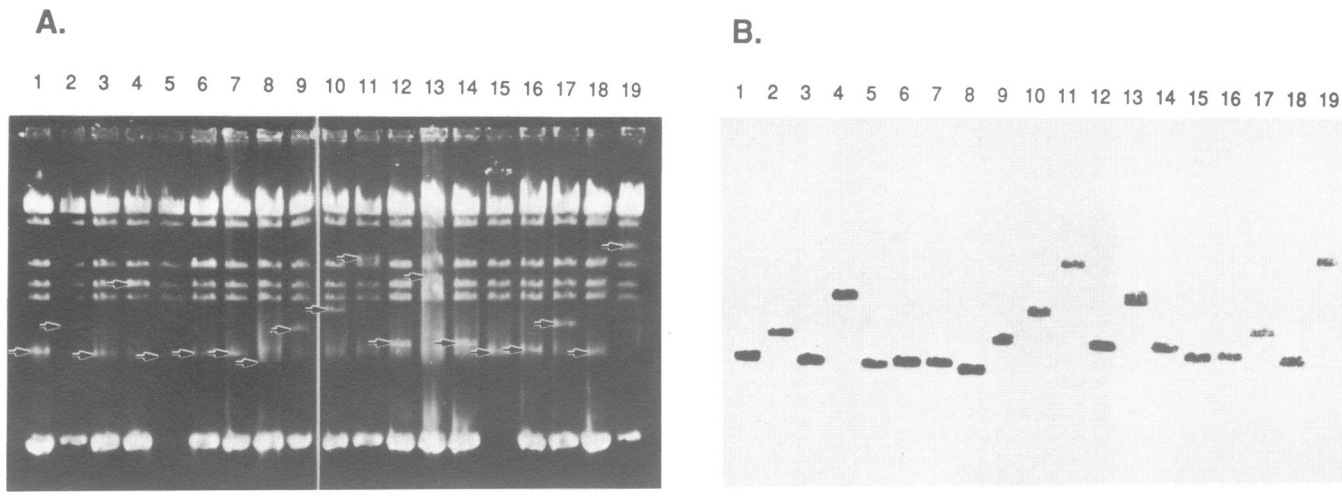

Figure 3. Physical characterization of the products of transformation-associated recombination between a human YAC12 and a DNA fragment containing an Alu sequence. In **A** the chromosomal size DNAs isolated from clones containing the original and recombinant YACs are ethidium bromide-stained. In **B** the YACs are identified with a labelled pBR322 probe. Lanes 1–18: randomly chosen *URA3–HIS3* recombinants between a human YAC12-A and an Alu-containing fragment. Lane 19: karyotype of the donor strain containing a YAC12.

Table 3. Recombination between an Alu-*HIS3*-telomere fragment and YACs containing human DNA in RAD$^+$ and *rad52* strains

YAC	YAC transformants (Ura$^+$)*	YACs that are containing *HIS3*	% recombinants among Ura$^+$ transformants
RAD$^+$			
360 kb YAC	2120 (149)**	135 (10)***	6.4
170 kb YAC	1120 (18)	12 (1)	1.0
510 kb YAC	1104 (240)	12 (3)	1.1
360 kb YAC (broken)	510 (117)	248 (5)	48.6
rad52			
360 kb YAC	880 (18)	5 (0)	0.6

*The YAC centromeric marker *URA3* was selected. The presence of the unselected YAC distal marker TRP1 or the fragment marker HIS3 was determined in the Ura$^+$ colonies.
** Number of transformants that were Ura$^+$ Trp$^-$. (In the transformants YACs have lost a telomeric arm).
***His$^+$ colonies that were also Trp$^+$.

end (39). This DNA fragment was added to the yeast chromosome mix at a ratio of approximately 100:1 plasmid to YAC. When the YAC centromeric marker *URA3* was selected, the levels of co-transformation by the unselected plasmid marker was 6% for the 360 kb YAC12 and 1% for the 170 and the 510 kb human YACs (Table 3). All the co-transformants contained a single chimeric YAC. Most of the YACs lacked the *TRP1* telomeric marker and were 80 to 260 kb shorter than the original (Fig. 3). A few co-transformants (14 among 159 analyzed) retained the YAC telomeric marker and were 5 kb larger than the original YAC. In these clones the fragment appeared to be integrated into the internal region of YAC.

The *RAD52* gene was found to play a prominent role in co-transformation by the Alu-containing fragment. The level of YAC-fragment recombination in the mutant was reduced over 10-fold as compared to RAD$^+$ (Table 3).

The frequency of co-transformation with the Alu-containing fragment in the RAD$^+$ strain was increased to nearly 50% when the YAC DNA was broken (Table 3). (Shearing of the YAC DNA was done by passing of the melted plug one time through a 1 ml pipette tip before transformation). All the co-transformants contained a single chimeric YACs that were 120 to 240 kb shorter than the original YAC12 (data not shown).

It is interesting that the shearing did not appear to affect the frequency of transformation. When broken YAC was used, the yield of Ura$^+$ transformants was the same as that observed for unbroken YAC although most of the transformants were Trp$^-$. All the Ura$^+$ Trp$^-$ transformants (20 among 20 analyzed) contained YACs that were 30–140 kb shorter than the parent YAC (data not shown). Transformants lacking a telomeric marker could have arisen by healing of the broken YAC ends at telomeric-like sequences frequently present in human DNA (53).

Co-transformants must have resulted from recombination between Alu's in the YACs and the fragment since there were none when a fragment was used that lacked the Alu sequence (data not shown). We propose that the interaction between a YAC and an Alu-containing fragment can be used as a model for YAC

Table 4. Mitotic recombination between homologous and heterologous * YACs in wild-type and *rad52* strains

Strain	Recombination** between YACs ($\times 10^{-3}$)			
	homologous YACs		heterologous YACs	
	360 kb/360 kb	170 kb/170 kb	360 kb/510 kb	360 kb/390 kb
RAD$^+$	6.8	6.0	0.32 (4)	0.18 (3)
rad52	1.4	ND***	ND	ND

*All YACs contain human DNA inserts except for the 390 kb mouse YAC.
**Recombination is either presented as rates for homologous YACs (see Material and Methods) or as the average frequency for heterologous YACs because of a low number of recombinants. In parentheses are presented the total number of recombinants detected for heterologous YACs.
***ND, not determined.

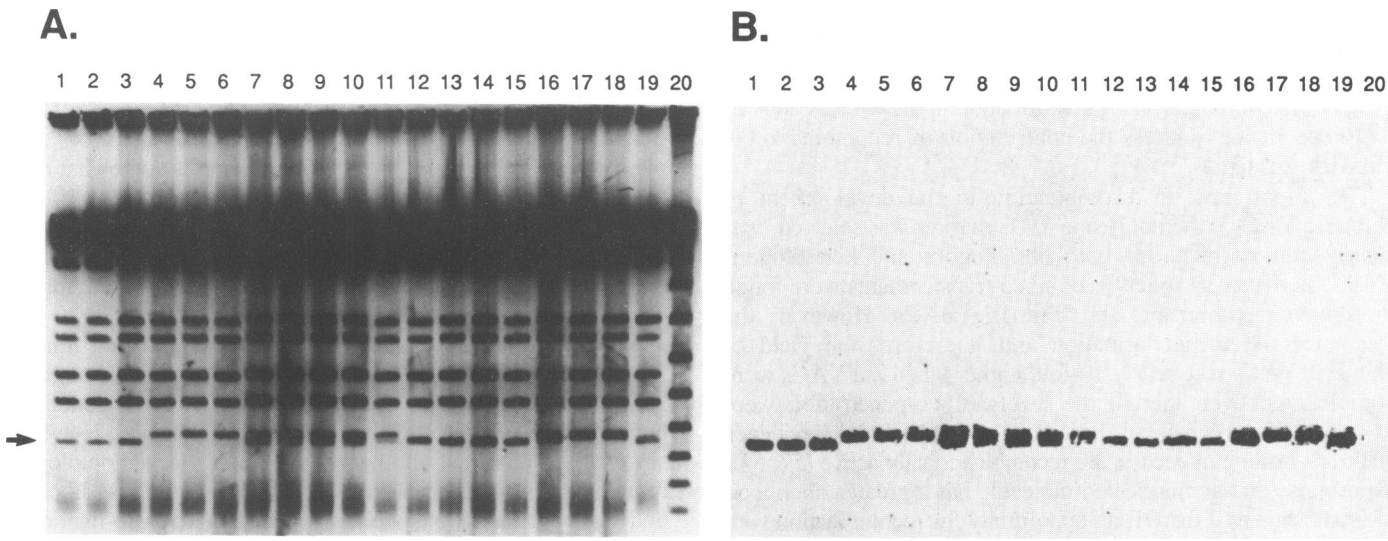

Figure 4. Physical characterization of the products of mitotic recombination of a pair of the 170 kb human homologous YACs. In **A** chromosomal size DNAs isolated from the clones containing the original and recombinant YACs are ethidium bromide-stained (the picture is a negative rather than a positive). In **B** the YACs are identified with a labelled pBR322 probe. Lanes 1–3, 12–15 and 19: eight randomly chosen *URA3–HIS3* recombinants between homologous human YACs. Lanes 4–6, 17–18: five randomly chosen *LYS2–TRP1* recombinants between homologous human YACs. Lanes 7–8: two *URA3–HIS3* recombinants and the original *LYS2–HIS3* YAC. Lanes 9–10: two *LYS2–TRP1* recombinants and the original *URA3–TRP1* YAC. Lane 11, 16: karyotype of the original strain containing YAC21-A and YAC21-B. Lane 20: l multimers ladder. Noted that a *LYS2–HIS3* derivative of YAC21 is about 20 kb bigger than a *URA3–TRP1* derivative. Arrows indicate approximate positions of YACs.

chimera formation during transformation. We note that these results are similar to those obtained when the Alu-containing vectors were targeted to established YACs in mitotic cells resulting in YAC fragmentation (12).

Recombination of YACs during mitotic growth

Formally the high level of recombinant YACs in co-transformants could have arisen during mitotic growth following transformation. The rates of recombination between homologous chromosomes in yeast, based on homozygosis of recessive genetic markers, is typically less than 10^{-5}/cell/generation (54). To investigate inter-YAC recombination in mitosis, strains were developed that contained pairs of differentially marked homologous and heterologous YACs (see Materials and Methods). Four pairs of YACs were analyzed (Table 4): two homologous (human:human) and two heterologous (human:human and human:mouse). Inter-YAC recombination was identified by loss of one of the distal telomeric markers, *HIS3* or *TRP1* (i.e., clones had the phenotypes either Lys$^+$ Ura$^+$ Trp$^+$ His$^-$ or Lys$^+$ Ura$^+$ His$^+$ Trp$^-$) and the appearance of the remaining distal marker on both YACs (see Material and Methods). This could have occurred by homozygosis of a distal marker during the G_2 phase of the cell cycle. Homologous YACs exhibited recombination rates several orders of magnitude higher than reported for homologous chromosomes. Inter-YAC recombination was decreased 5-fold in a *rad52* mutant strain (Table 4). Nonhomologous YACs also appeared to be recombination-prone since they exhibited frequencies of recombination that are only 20 to 30-fold lower than observed with homologous YACs (Table 4).

Since human DNA contains many types of repeated sequences including Alu's, whose average separation is 4 kb (55), they could be an important component in a high level of recombination, resulting in unequal exchanges and changes in YAC lengths. We examined 60 Lys$^+$ Trp$^+$ and 56 Ura$^+$ His$^+$ recombinant clones derived from the strains containing the pairs of homologous YACs. For homologous YACs, the recombinants were the same

size as the original YACs, (Fig. 4), whereas for nonhomologous YACs the recombinants differed in size from the parent YACs (data not shown).

DISCUSSION

Mammalian DNAs contain a large number of repeats of sufficient length that they could be substrates in recombination. For example the frequencies of Alu's (~300 bp) and Line's (~5 kb) are approximately 1 per 4 kb or 100 kb of human DNA, respectively. These, along with the many pseudogenes and other categories of repeat sequences, could be important sources of recombination leading to chimeric YACs. The present study has enabled us to examine the efficiency of chimera formation by recombination between homologous DNAs and heterologous DNAs containing repeat sequences both during transformation and mitotic growth. While chimeric YACs can arise by co-ligation during the development of YAC libraries [as discussed by Burke (56)], the present system, based on retransformation of YACs, has allowed us to specifically address the contribution of recombination to chimera formation.

The importance of recombination in the development of chimeric YACs, when only one YAC marker was selected, was clearly demonstrated for both homologous and heterologous YACs. Between 30 and 50% of all co-transformants were found to contain recombinant, i.e., chimeric YACs. However, the frequency of co-transformation and, therefore, the yield of chimeric YACs was nearly 10-fold higher when the YACs were homologous, even though the levels of co-penetration were expected to be comparable. Based on the results and the evidence that transforming molecules are recombinationally active (57, 58), we propose that an unselected molecule has a greater likelihood of establishment if there is an opportunity for recombination with a selected molecule. This is obvious for the case when the molecules become physically linked (i.e., chimeras). The higher level of co-transformation for homologous as compared to heterologous YACs, even when they are not physically linked, might be due to greater opportunities for recombinational repair (similar to repair of ionizing radiation damage between YACs in mitotic cells) (59).

Lesions in DNA, particularly single and double-strand breaks which can lead to transformation-associated recombination (57, 58), are likely to be frequent in the transforming human DNA used for libraries. Many lesions would be close to commonly occurring repeats such as Alu's. Therefore, we investigated transformation-associated interactions between YACs and nonreplicating fragments that contained an Alu and a telomere. Co-transformation for the unselected fragment was as high as 6%. As expected, all co-transformants were recombinant and the recombination was *rad52* dependent. When the transforming YACs were broken, the level of recombination increased to approximately 50%, while the frequency of YAC transformation did not change. This indicates that broken ends are highly reactive in generating chimeric molecules.

Thus, recombination could be an important contributor to the formation of YAC chimeras. As we have shown, the conditions used for the development of YAC libraries often lead to co-penetration. Under these conditions, heterologous molecules could also undergo recombination and the repeated sequences along with DNA lesions are likely sources of recombination. Even if the repeats are diverged, they could mediate transformation-associated recombination (57, 58, 60). Since the likelihood of recombination would increase in the regions of greater homology, we suggest that there would be an enrichment for chimeras involving related DNAs such as pseudogenes.

Can transformation-associated recombination account for all chimera formation given that some libraries contain as much as 60–80% chimeric YACs? This would depend in part on the incidence of co-penetration. It is possible to estimate a minimum level of co-penetration of chromosome-size DNAs under the specific conditions used in the present experiments. For YACs and chromosomes in the range of 170 to 360 kb, the minimum frequency of co-transformation was between 3 and 5% under conditions where each DNA represented 3% (32 yeast chromosomes and two YACs) of the DNA molecules in the transformation mix. This suggests that there is a high level of co-penetration of chromosome size molecules. If co-penetration is equally likely for all molecules, more than 90% of transformants would be expected to contain at least two chromosomal size DNAs. [Such high frequency of co-transformation has been previously demonstrated for small plasmid DNAs (61).] Even if this estimation is somewhat high, since small and large molecules may penetrate with different efficiencies (5, 42), co-penetration by chromosomal size molecules is not likely a limiting factor for chimeric YAC formation under conditions that are commonly used for the development of YAC libraries.

Since there is frequent co-penetration, a significant limiting factor of chimera formation during co-transformation might be lack of homology. As we have shown, YACs containing homologous DNAs frequently undergo recombination. However, since most DNAs in a library will not contain large homologous regions and since heterologous DNAs are much less likely to undergo recombination, we suggest that *in vitro* co-ligation is an important factor in the formation of chimeric molecules during the development of YAC libraries.

The role of co-ligation in chimeric YAC formation during the construction of a human library was recently examined by Wada *et al.* (34). To minimize opportunities for co-ligation, the ends of restricted DNA were partially filled-in. Using a 1:1 mixture of human:mouse DNAs, they observed no co-cloned interspecies DNAs. However, there was a high level of YACs that contained sequences that could hybridize to more than one human chromosome. It was proposed that chimeric clones generally arise by a mechanism that does not involve *in vitro* co-ligation. While their results demonstrate that intraspecies DNA can recombine efficiently during transformation, there is an alternative explanation which is specifically addressed in the present studies. Under the conditions of limited ligation, many molecules would lack telomeres. As indicated in their experiments, and also demonstrated in the present work, co-penetration may have been frequent. The opportunity for YAC establishment is greatly enhanced (as much as 10-fold) when there is co-penetration by homologous as compared to heterologous YACs under conditions where one YAC marker is selected. Because few molecules under the conditions used would have been expected to have telomeres at both ends (thus most molecules have broken ends), functional YACs might be expected to arise primarily by recombination between co-penetrating molecules when two telomeric markers are selected. Thus, there may be enrichment for human:human chimeras containing regions of homologous or related DNAs. Molecules with little or no homology would recombine much

less frequently. Thus, while both can occur, the relative role of *in vitro* ligation vs *in vivo* recombination during development of YAC libraries still needs to be established.

While mitotic recombination could contribute to the formation of YAC chimeras between heterologous pairs of YACs, we demonstrated that levels of inter-YAC recombination during mitosis are too low to play a significant role in the formation of YAC chimeras. It is interesting, however, that homologous YACs exhibit mitotic recombination levels nearly 100-fold higher than natural yeast chromosomes. These high levels could be due to differences in the organization of human DNAs, including multiple large repeats such as Alu and Line sequences, origins of replication and chromatin distribution. We suggest that spontaneous mitotic recombination may be a useful alternative for isolating recombinants between overlapping YACs (14).

This study complements previous investigations into how the *RAD52* gene product affects the integrity of cloned human DNA. Deletion of this gene results in greatly reduced transformation-associated recombination in plasmids and deletions in YACs (21, 22, 58). Furthermore, we have demonstrated that while *rad52* mutant strains may exhibit mutator activity (62, 63), they do not exhibit higher levels of mutations in transforming chromosomal DNA (Larionov *et al.*, submitted for publication). The *rad52* mutation also decreases the level of internal deletions and rearrangements in mammalian YACs during mitotic propagation (22, 64). The present results demonstrate another feature of the utility of a *rad52* mutant for cloning of human DNAs, namely a reduction in chimeric YACs during co-transformation. These data obtained with model systems are consistent with recent observations of reduced numbers of chimeric clones in the development of a human library (65, 66). While the frequency can be reduced using a *rad52* mutant host strain, it may be possible to reduce further the incidence of chimeric YACs arising by recombination. This is the subject of continuing investigations into the genetic controls of YAC integrity.

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REFERENCES

1. Burke, D. T., Carle, G. F., and Olson, M. V. (1987) *Science* 236, 806–812.
2. Brownstein, B. H., Silverman, G. A., Little, R. D., Burke, D. T., Korsmeyer, S. J., Schlessinger, D., and Olson, M. V. (1989) *Science* 244, 1348–1351.
3. McCormick, M. K., Shero, J. H., Cheung, M. C., Kan, Y. W., Hieter, P., and Antonarakis, S. A. (1989) *Proc. Natl. Acad. Sci. USA* 86, 9991–9995.
4. Abidi, F. E., Wada, M., Little, R. D., and Schlessinger, D. (1990) *Genomics* 7, 363–376.
5. Albertsen, H. M., Abderrahim, H., Cann, H. M., Dausset, J., Le Paslier, D., and Cohen, D. (1990) *Proc. Natl. Acad. Sci. USA* 87, 4256–4260.
6. Anand, R., Rilley, J. H., Butler, R., Smith, J. C., and Markham, A. F. (1990) *Nucl. Acids Res.* 18, 1951–1956.
7. Imai, T., and Olson, M. V. (1990) *Genomics* 8, 297–303.
8. Larin, Z., Monaco, A. P., and Lehrach, H. (1991) *Proc. Natl. Acad. Sci. USA* 88, 4123–4127.
9. Rossi, J. M., Burke, D. T., Leung, J. C. M., Koos, D. S., Chen, H., and Tilghman, S. M. (1992) *Proc. Natl. Acad. Sci. USA* 89, 2456–2460.
10. McCormick, M. K., Buckler, A., Bruno, W., Campbell, E., Shera, K., Torney, D., Deaven, L., and Moyzis, R. (1993) *Genomics* 18, 553–558.
11. Kusumi, K., Smith, J. S., Segre, J. A., Koos, D. S., and Lander, E. S. (1993) *Mamm.. Genome* 4, 391–392.
12. Pavan, W. J., Hieter, P., and Reeves, R. H. (1990) *Proc. Nat. Acad. Sci. USA* 87, 1300–1304.
13. Campbell, C., Gulati, R., Nandi, A. K., Floy, K., Hieter, P., and Kucherlapati, R. S. (1991) *Proc. Natl. Acad. Sci. USA* 88, 5744–5748.
14. Green, E. D., and Olson, M. V. (1990) *Science* 250, 94–98.
15. Ragoussis, J., Trowsdale, J., and Markie, D. (1992) *Nucl. Acids Res.* 20, 3135–3138.
16. Den Dunnen, J. T., Grootscholten, P. M., Dauwerse, J. G., Walker, A. P., Monaco, A. P., Butler, R., Anand, R., Coffey, A. J., Bentley, D. R., Steensma, H. Y. and Van Ommen, G. J. B. (1992) *Hum. Mol. Genet.* 1, 19–28.
17. Romoto, F., and Carle, G. F. (1994) *Nucl. Acids Res.* 22, 1208–1214.
18. Pavan, W. J., Hieter, P., and Reeves, R. H. (1990) *Mol. Cell. Biol.* 10, 4163–4169.
19. Pachnis, V., Pevny, L., Rothstein, R., and Costantini, F. (1990) *Proc. Nat. Acad. Sci. USA.* 87, 5109–5113.
20. Gnirke, A., Huxley, C., Peterson, K., and Olson, M. V. (1993) *Genomics* 15, 659–667.
21. Neil, D. L., Villasante, A., Fisher, R. B., Vetrie, D., Cox, B., and Tyler-Smith, C. (1990) *Nucl. Acids Res.* 18, 1421–1428.
22. Kouprina, N., Eldarov, M., Moyzis, R., Resnick, M., and Larionov, V. (1994) *Genomics* 21, 7–17.
23. Schlessinger, D. (1990) *Trends in Genetics* 6, 248–258.
24. Bronson, S. K., Pei, J., Taillon-Miller, P., Chorney, M. J., Geraghty, D. E., and Chaplin, D. D. (1991) *Proc. Natl. Acad. Sci. USA* 88, 1676–1680.
25. Bates, G. P., Valdes, J., Hummerich, H., Baxendale, S., Le Paslier, D. L., Monaco, A. P., Tagle, D., MacDonald, M. E., Altherr, M., Ross, M., Brownstein, B. H., Bentley, D., Wasmuth, J. J., Gusela, J. F., Cohen, D., Collins, F., and Lehrach, H. (1992) *Nature Genetics* 1, 180–187.
26. Green, E. D., Riethman, H. C., Dutchik, J. E., and Olson, M. V. (1991) *Genomics* 11: 658–669.
27. Schlessinger, D., Little, R. D., Freije, D., Abidi, F., Zucchi, I., Porta, G., Pilia, G., Nagaraja, R., Johnston, S. K., 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.
28. Silverman, G., Jockel, J., Domer, P., Mohr, R., Taillon-Miller, P., and Korsmeyer, S. (1991) *Genomics* 9, 219–228.
29. Little, R., Pilia, G., Johnston, S., D'Urso, M., and Schlessinger, D. (1992). *Proc. Natl. Acad. Sci. USA* 89, 177–181.
30. Monaco, A. P., Walker, A. P., Millwood, I., Larin, Z., and Lehrach, H. (1992) *Genomics* 12, 465–473.
31. Selleri, L., Eubanks, J. H., Giovannini, M., Hermanson, G. G., Romo, A., Djabali, M., Maurer, S., McElligott, D. L., Smith, M. W., and Evans, G. A. (1992) *Genomics* 14, 536–541.
32. Sleister, H. M., Mills, K. A., Blackwell, S. E., Killary, A. M., Murray, J. C., and Malone, R. E. (1992) *Nucl. Acids Res.* 20, 3419–3425.
33. Foote, S., Vollrath, D., Hilton, A., and Page, D. C. (1992) *Science* 258, 60–66.
34. Wada, M., Abe, K., Okumura, K., Taguchi, H., Kohno, K., Imamoto, F., Schlessinger, D., and Kuwano, M. (1994) *Nucl. Acids Res.* 22, 1561–1554.
35. Sherman, F., Fink G. R., and Lawrence, C. W. (1979) Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
36. Rothstein, R. J. (1983) *Meth. Enzymol.* 101, 202–211.
37. Sears, D. D., Hegemann, J. H., and Hieter, P. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5296–5300.
38. Shero, J. H., Koval, M., Spencer, F., Palmer, R., Hieter, P., and Koshland, D. (1991) *Methods Enzymol.* 194, 749–773.
39. Pavan, W. J., Hieter, P., Sears, D., Burkhoff, A., and Reeves, R. (1991) *Gene* 106, 125–127.
40. Burgers, P. M. J., and Percival K. J. (1987) *Anal. Biochem.*. 163, 391–397.
41. McCormick, M. K., Shero, J. H., Connelly, C., Antonarakis, S., and Hieter, P. (1990) *Technique* 2, 65–71.
42. Connelly, C., McCormick, M. K., Shero, J., and Hieter P. (1991) *Genomics* 10, 10–16.
43. Carle, G., and Olson, M. (1984) *Nucl. Acids Res.* 12, 5647–5664.
44. Boeke, J. D., Lacroute, F., and Fink, G. R. (1984) *Mol. Gen. Genet.* 197, 345–346.
45. Chattoo, B. B., Sherman, F., Azubalis, D. A., Fjellstedt, T. A., Mehnert, D., and Ogur, M. (1979) *Genetics* 93, 51–65.

46. Lea, D. E., and Coulson, C. A. (1949) *J. Genet.* 49, 264–284.
47. Ma, H., Kunes, S., Schatz, P. J., and Botstein, D. (1987) *Gene* 58, 201–216.
48. Kozhina, T. N., Peshekhonov, V. T., and Chepurnaya, O. V. (1988) *Genetika* (Russ.) 24, 993–997.
49. Erickson, J. R., and Johnston, M. (1993) *Genetics* 134, 151–157.
50. Resnick, M. A., and Martin, P. (1976) *Mol. Gen. Genet.* 143, 119–129.
51. Klein, H. L. (1988) *Genetics* 120, 367–372.
52. Thomas, B. J. and Rothstein, R. (1989) *Genetics* 123, 725–738.
53. Riethman, H. C., Moyzis, R. K., Meyne, J., Burke, D. T., and Olson, M. V. (1989) *Proc. Natl. Acad. Sci. USA* 86, 6240–6244.
54. Hartwell, L. H., and Smith, D. (1985) *Genetics* 110, 381–395.
55. Moyzis, R. K., Torney, D. C., Meyne, J., Buckingham, J. M., Wu, J.-R., Burks, C., Sirotkin, K. M., and Goad, W. B. (1989) *Genomics* 4, 273–289.
56. Burke, D. T. (1990) *GATA* 7, 94–99.
57. Mezard, C., Pompon, D., and Nicolas, A. (1992) *Cell* 70, 659–670.
58. Larionov, V., Kouprina, N., Eldarov, M., Perkins, E., Porter, G., and Resnick, M. A. (1994) *Yeast* 10, 93–104.
59. Resnick, M. A., Zgaga, Z., Fogel, S., Hieter, P., Westmoreland, J., Fogel, S., and Nilsson-Tillgren, T. (1992) *Mol. Gen. Genet.* 234, 65–73.
60. Priebe, S. D., Westmoreland, J., Nilsson-Tillgren, T., and Resnick, M. A. (1994) *Mol. Cell. Biol.*, in press.
61. Yamamoto, T., Moerschell, R. P., Wakem, L. M., Ferguson, D., and Sherman, F. (1992) *Yeast* 8: 935–948.
62. von Borstel, R. C., Cain, K. T., and Steinberg, C. M. (1971) *Genetics* 69, 17–27.
63. Kunz, B. A., Peters, M. G., Kohalmi, S. E., Armstrong, J. D., Glattke, M., and Badiani, K. (1989) *Genetics* 122, 535–542.
64. Dunford, R., Vilageliu, L., and Moore, G. (1993) *Plant Mol. Biol.* 21, 1187–1189.
65. Chartier, F. L., Keer, J. T., Sutchliffe, M. J., Henriques, D. A., Mileham, P., and Brown, S. D. M. (1992) *Nature Genetics* 1, 132–136.
66. Ling, L. L., Ma, N., S.-F., Smith, D. R., Miller, D. D., and Moir, D. T. (1993) *Nucl. Acids Res.* 21, 6045–6046.